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(72) Inventors: MEZES, Peter, S. ; 5101 Oakridge Drive, Midland, MI 48640 (US). RICHARD, Ruth, A. ; 5111 Plainfield Street, Midland, MI 48640 (US). AFFHOLTER, Joseph, A. ; 823 E. Sanford Road, Midland, MI 48640 (US). KOTITE, Nicolas, J. ; 5704 Woodbridge Lane, Midland, MI 48640 (US).

(54) Title: DIMER AND MULTIMER FORMS OF SINGLE CHAIN POLYPEPTIDES

CONSTRUCTION OF *E.COLI* SCFV EXPRESSION PLASMIDS CONTAINING HUM V$_L$IV

(57) Abstract

The present invention discloses novel proteins which are dimers and multimers of single chain antibodies. The single chain antibodies contain polypeptides of two domains derived from the variable domains of antibodies which are joined by a peptide linker. The dimers and multimers are formed by non-covalent linking of the single chain polypeptides. Uses include all those appropriate for monclonal and polyclonal antibodies and fragments thereof, including use as a bispecific antigen-binding molecule.

DIMER AND MULTIMER FORMS OF SINGLE CHAIN POLYPEPTIDES

This invention relates to the field of molecular biology and production of single chain polypeptides.

5   The basic structure of an antibody is a tetramer composed of two identical heterodimers each consisting of a light and a heavy chain. The antibody molecule is divided into variable (V) domains that are responsible for binding specificity and constant (C) domains that carry out various effector functions. The V domain is constructed of one V homology unit from both the light and heavy chains, designated V_L and V_H respectively. The variable region

10   can be further subdivided into framework regions (FR) and complementarity determining regions (CDR), also designated as hypervariable regions. The FR maintain the structural integrity of the variable region domain. The CDR are the polypeptide segments within the variable region that mediate binding to the antigen. As the antibody contains two heterodimers, it has two antigen binding sites (divalent).

15   By using current techniques in molecular biology, it is possible to clone polypeptide chains. The more complex the molecules, e.g., the multichain immune receptors of the Ig superfamily, the more difficult they are to produce in a single foreign host. Genes coding for each chain of a complex molecule can be cloned and expressed in separate hosts but the aggregation and refolding of the resultant polypeptides into a biologically active entity is

20   difficult to achieve. Multiple chains expressed by multiple genes within the same host have an advantage in aggregating and refolding into native structure but their expression in stoichiometric amounts is difficult to regulate. Thus, neither approach has proven to be efficient.

Since it is the variable regions of light and heavy chain antibodies that interact

25   with an antigen, single chain polypeptides have been created with one V_L and one V_H joined by a peptide linker (U.S. Patent 4,946,778). This single chain fragment (Fv) is univalent and is one of the smallest structures necessary for antigen binding activity having all 6 CDR regions of a V_L-V_H combination.

The use of the functional smaller polypeptides which maintain an antigen

30   recognition site are also advantageous when used for therapeutic purposes in mammals in that the smaller molecules are capable of rapidly localizing in the target tissue, such as single chain antibodies localizing in cancerous tissue. However, while being able to penetrate the desired tissue, polypeptides with a molecular weight of less than about 50,000 daltons have the disadvantage of being retained within the glomerulus of the kidney This is particularly

35   disadvantageous when the polypeptide is bound with a carrier, such as a radioisotope or toxin, as the isotope or toxin will also accumulate in the kidneys.

Single chain polypeptides also suffer from the disadvantage of having only one binding site, thereby reducing their avidity.

It would therefore be advantageous to obtain constructions of the single-chain antibodies which retain their antigen or ligand recognition properties, are of sufficient molecular weight to enhance retention at the target site with reduced retention in the kidney, and have a multiplicity of binding sites to enhance the avidity of the polypeptide. In addition,

5 it would be beneficial if the single chain immunoglobulin could be rendered bispecific to allow for recognition of different epitopes on the target tissue (or lymphocyte) or to allow for antibody-based recruitment of other immune effector functions (i.e., complement proteins, cytotoxic lymphocytes, etc.).

Surprisingly it has been found that single chain antibodies will non-covalently

10 associate to form dimers and multimers. In one embodiment, the invention is a protein comprising a dimer or multimer of single chain polypeptides wherein the polypeptides are non-covalently linked and each polypeptide comprises (a) a first polypeptide comprising an antigen binding portion of a variable domain from an immunoglobulin; (b) a second polypeptide comprising an antigen binding portion of a variable domain from an

15 immunoglobulin; and (c) a peptide linker linking the first and second polypeptides (a) and (b) into a single chain polypeptide having affinity for an antigen.

In another embodiment, the invention is a protein comprising a dimer or multimer of single-chain polypeptides wherein the polypeptides are non-covalently linked and each polypeptide comprises (a) a first polypeptide comprising an antigen binding portion of a

20 variable domain from an immunoglobulin; and (b) a second polypeptide comprising an antigen binding portion of a variable domain of an immunoglobulin wherein polypeptides (a) and (b) are joined by a peptide bond.

In another aspect the present invention includes the aforementioned dimer or multimers conjugated to an imaging marker or therapeutic agent. The invention also includes

25 a composition comprising the dimer or multimer conjugated to an imaging marker or therapeutic agent in a pharmaceutically acceptable carrier.

The invention is also directed to a method for *in vitro* or *in vivo* diagnostics of cancer which comprises administering to an animal containing a tumor expressing a recognizable marker, a pharmaceutically effective amount of a composition containing a dimer

30 or multimer of single chain polypeptides wherein the first and second polypeptide are specific for the recognizable marker.

In another embodiment, the invention is a method for intraoperative therapy which comprises (a) administering a pharmaceutically effective amount of the aforementioned composition containing di- or multimers of a single chain antibody against a recognizable

35 marker to a patient containing a tumor expressing this recognizable marker, whereby the tumor is localized and (b) excising the localized tumor.

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In still another embodiment, the invention concerns a process f r preparing and expressing the aforementioned dimer and multimers of single chain polypeptides. Such an exemplary process comprises:

   (i)   providing a genetic sequence coding for a single chain polypeptide;

5 (ii)   transforming a host cell with the genetic sequence;

   (iii)   expressing the genetic sequence in the host cell; and

   (iv)   recovery of single chain polypeptides which have non-covalently linked to form dimers and multimers.

Brief Description of the Drawings

10   Figure 1 shows the digestion of pRW83 to produce vectors used in the construction of plasmid pSCFV31.

         Figure 2 shows the construction of plasmid pSCFV31.

         Figure 3 illustrates the nucleotide sequence and amino acid sequence of SVFV1.

         Figures 4 shows the construction of plasmid pSCFV UNIH.

15   Figure 5 illustrates the nucleotide sequence and amino acid sequence of Hum4 V_L gene Cla I-Hind III segment in pRL1001.

         Figure 6A illustrates the plasmid pSCFV VHH and plasmid pSCFV UHM. (B) and (C) illustrate a schematic representation of a single chain Fv monomer and single chain Fv dimer, respectively. The darkened areas represent the complementarity determining regions.

20   Figure 7 illustrates the nucleotide sequence and amino acid sequence of a Human Subgroup 4 variable light chain (H4V_L) and a murine variable having chain single chain Fv immunoglobulin fragment.

         Figure 8 illustrates the DNA sequence and amino acid sequence of CC49 SCFV species with a pI of 8.1(A), 5.8(B) and 5.2(C).

25   Figure 9 shows the results of a cation exchange chromatograph activity, SDS-PAGE and Western Blot of E. coli pSCFV UHM8.1 periplasmic fraction.

         Figure 10 shows the results of an anion exchange chromatograph, SDS-PAGE and Western Blot of Pool 2 (Figure 9) of E coli pSCFV UHM8.1 periplasmic fraction.

         Figure 11 shows the results of a gel filtration chromatograph SDS-PAGE and
30 isoelectric focusing (IEF) from fraction 1 of the anionic exchange (Figure 10).

         Figure 12 shows the results of a gel filtration chromatograph, activity SDS-PAGE and Western Blot of E. coli pSCFV UHM5.2 periplasmic fraction.

         Figure 13 illustrates the results of a CC49 antibody competition based on moles of antibody binding sites.

35   Figure 14 illustrates the results of a competition ELISA read one hour after addition of substrate.

         Figure 15 shows the construction of plasmids pP41, pP42, pP421, pP422, pP431 and pP432.

Figure 16 illustrates the DNA sequence and amino acid sequence of CC49 SCFV in pPY21(A) and pPY22(B).

Figure 17 shows the relative activity of CC49 SCFV species obtained fr m Pichia pP431 and pP432 clones.

Figure 18 shows the results of gel filtration and SDS-PAGE analysis of Pichia CC49 scFv species having a pl of 5.2.

Figure 19 shows the results of an anion exchange chromatography and SDS-PAGE of Pichia CC49 scFv species having a pl of 5.2.

Figure 20 shows the results of an isoelectric focusing and Western Blot of Pichia scFv species having a pl of 5.2.

Figure 21 illustrates the results of a competition ELISA based on (A) moles of antibody sites and (B) moles of antibody.

Figure 22 shows the construction of plasmids pATDFLAG and pSC49FLAG.

Figure 23 illustrates the nucleotide sequences and amino acid sequences in plasmid pATDOFLAG.

Figure 24 illustrates the nucleotide sequences and amino acid sequences in plasmid pSC49FLAG.

Figure 25 shows the results of an M2 affinity chromatograph (A), analytical gel filtration (B), SDS-PAGE (C) and Western Blot of H4L 49HF scFv's.

Figure 26 illustrates the results of binding of H4L49HF single chain species by TAG ELISA (A, B and C) or BSM ELISA (D).

Figure 27 illustrates the results of a competition assay based on moles of antibody binding sites of H4L49F single chain species.

Figure 28 illustrates the results of a competition assay based on moles of H4L49F single chain species.

Nucleic acids, amino acids, peptides, protective groups, active groups and such, when abbreviated, are abbreviated according to the IUPAC IUB (Commission on Biological Nomenclature) or the practice in the fields concerned.

As used herein, the term "non-covalently linked" means a polypeptide that will be separated on the basis of its molecular weight by size exclusion chromatography when done under non-reducing or reducing conditions, but will dissociate into two or more polypeptides when subjected to denaturing conditions such as heating and sodium dodecyl sulfate.

The term "single chain polypeptide" as used herein means a polypeptide from two original peptides linked by a peptide linker where the two original peptides are domains from an antibody and is capable of binding an antigenic determinant or receptor. "Domain" is a segment of protein that assumes a discrete function, such as antigen binding or antigen recognition.

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A "dimer" is two single chain polypeptides which are non-coval ntly linked and the term "multimer" means an even number of dimers which are non-c valently linked. "Vector" means a DNA element used as a vehicle for cloning a fragment of foreign DNA.

The single chain polypeptides which form dimers and multimers in the present invention can be obtained from any member immunoglobulin (Ig) family, including IgG, IgM, IgE, IgD and IgA. The two peptides in the single chain are generally selected from a light and heavy chain variable region of an antibody. Alternatively, it may be desirable to produce dimers and multimers of single polypeptides where the two original peptides are derived from the same domain (e.g., V_L-linker-V_L or V_H-linker-V_H). The present invention also includes the formation of dimers and multimers wherein one single chain polypeptide is directed against one antigen and the second or additional polypeptides are directed against a different antigen.

The dimers and multimers of the present invention formed by domains of the antibody are advantageous for therapeutic purposes in mammals as they retain their antigenic recognition properties, contain multiple binding sites and are of larger molecular weight than the single chain monomers, thereby reducing their retention in the kidneys.

Isolation of an Appropriate DNA Sequence

To prepare a vector containing the DNA sequence for an antigen binding portion of an Ig superfamily protein, a source of the genes encoding for these regions will be required. The appropriate DNA sequence can be obtained from published sources or can be obtained by standard procedures known in the art. For example, Kabat et al., Sequences of Proteins of Immunological Interest 4th ed., (1991), published by The U.S. Department of Health and Human Services, discloses sequences of most of the Ab variable regions which have been described to date. WO 90/04410 published May 3, 1990 discloses a DNA sequence for variable regions of antibodies against tumor associated antigen (TAG).

When the genetic sequence is unknown, it is generally possible to utilize cDNA sequences obtained from mRNA by reverse transcriptase mediated synthesis as a source of DNA to clone into a vector. For antibodies, the source of mRNA can be obtained from a wide range of hybridomas. See, for example, the catalogue ATCC Cell Lines and Hybridomas, American Type Culture Collection, 20309 Parklawn Drive, Rockville Md., USA (1990). Hybridomas secreting monoclonal antibodies reactive with a wide variety of antigens are listed therein, are available from the collection, and usable in the present invention. These cell lines and others of similar nature can be utilized as a source of mRNA coding for the variable region or to obtain protein to determine amino acid sequence from the monoclonal antibody itself. The specificity of the antibody to be engineered will be determined by the original selection process. The class of antibody can be determined by criteria known to those skilled in the art.

Variable regions of antibodies can also be derived by immunizing an appropriate vertebrate, normally a domestic animal, and most conveniently a mouse. The immunogen will

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be the antigen of interest, or where a hapten, an antigenic conjugate of the hapten to an antigen such as keyhole limpet hemocyanin (KLH). The immunization may be carried ut conventionally with one or more repeated injections of the immunogen into the host mammal, normally at two to three week intervals. Usually three days after the last challenge, the spleen

5   is removed and dissociated into single cells to be used for cell fusion to provide hybridomas from which mRNA can readily be obtained by standard procedures known in the art.

From cultured lymphocytes or hybridomas, mRNA can be obtained which in turn is utilized to produce cDNA. From the pool of cDNA, the desired cDNA can be amplified using such techniques as the polymerase chain reaction (PCR) or PCR with splicing by overlap

10   extension (PCR-SOE). PCR in essence involves exponentially amplifying DNA in vitro using sequence specified 5' and 3' oligonucleotides. PCR is described in Mullins et al., *Meth. Enz.*, 155, 335-350 (1987); and *PCR Technology*, Erlich (ed.) (1989).

When an antibody of interest is obtained, and only its amino acid sequence is known, it is possible to reverse translate the sequence.

15   To form the single chain polypeptides which form dimers and multimers it is generally necessary to have a suitable peptide linker which links the first and second polypeptides. Suitable linkers for joining the polypeptides are those which allow the separate polypeptide chains to fold into a single polypeptide chain which will have a three dimensional structure very similar to the original structure made of two polypeptide chains, and allows

20   non-covalent linkage of single chains to form dimers and multimers of the single polypeptide chain. The linker must thus be (1) sufficiently large to span the distance between the two polypeptides; (2) sufficiently flexible to allow the association of the two polypeptides; and (3) relatively hydrophilic, being on the water-accessible surface of the molecule. Obtaining a three dimensional structure similar to the original antibody molecule allows the resulting

25   single chain polypeptide, dimer or multimer to recognize and react with the initial antigen. Generally, a linker which allows separate polypeptide chains to fold into a single polypeptide chain with a three dimensional structure similar to the original structure made of two polypeptide chains should also allow formation of dimer and multimers. Linkers having the desired properties can be obtained by the method disclosed in U.S. Patent 4,946,778, the

30   disclosure of which is hereby incorporated by reference. From the polypeptide sequences generated by the methods described in the 4,946,778, genetic sequences coding for the polypeptide can be obtained.

It is also necessary that the linker peptide be attached to the peptides such that the binding of the linker to the individual polypeptides does not interfere with the binding

35   capacity of the antigen recognition site.

A preferred linker is the helical linker designated 205 as disclosed in Pantoliano et al. *Biochem.*, 30, 10117-10125 (1991).

Another preferred linker is 205C, which has the amino acid sequence of Leu Ser Ala Asp Asp Ala Lys Lys Asp Ala Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys Asp Leu.

The linker is generally about 10 to about 50 amino acid residues. Preferably the linker is about 10 to about 30 amino acid residues. More preferably the linker is about 12 to

5   about 30 amino acid residues. Most preferred is a linker of about 15 to about 25 amino acid residues.

In another embodiment, the invention comprises two variable domains joined by a peptide bond without an intervening linker polypeptide. Examples include V_L-V_L, V_H-V_H, V_L-V_H and V_H-V_L single chain polypeptides. Such constructs would maximize inter-polypeptide

10   chain interactions to form multimers while minimizing the potential for intra-polypeptide chain interactions to form monomers.

Expression vehicles for production of the molecules of the invention include plasmids or other vectors. In general, such vectors contain replicon and control sequences which are derived from species compatible with a host cell. The vector ordinarily carries a

15   replicon site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. For example, E. coli is readily transformed using pBR322 [Bolivar et al., Gene, 2, 95- (1977), or Sambrook et al., Molecular Cloning, Cold Spring Harbor Press, New York, 2nd Ed. (1989)], a plasmid derived from an E. coli species which contains genes for ampicillin and tetracycline resistance, and thus provides an easy means for identifying transformed cells.

20   Expression vectors compatible with procaryotic cells are well known in the art and are available from commercial sources, e.g., pBR325 from Gibco Biological Research Laboratories. Typical of vector plasmids suitable for procaryotic cells are pUC8, pUC9, pBR322 and PBR329 available from Biorad Laboratories (Richmond, CA) and pPL and pKK222 available from Pharmacia-LKB (Piscataway, NJ), and the Bluesript SK/KS vectors from Stratagene (LaJolla, CA).

25   Plasmids suitable for eukaryotic microbes may also be used. S. cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms although a number of other strains, such as Pichia pastoris, are available. Cultures of cells derived from multicellular organisms such as Sp2/0 or Chinese Hamster Ovary (CHO), which are available from the ATCC, may also be used as hosts. Typical of vector plasmids suitable for eukaryotic

30   cells are pSV2neo and pSV2gpt (ATCC); pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnology, Inc.).

The use of viral expression vectors to express the genes for polypeptides of the present invention is also contemplated. As used herein, the term "viral expression vector" refers to a DNA molecule that includes a promoter sequence derived from the long terminal

35   repeat (LTR) region of a viral genome. Exemplary phage include λ phage and fd phage (See, Sambrook et al., supra; McCafferty et al., Nature, 6301, 552-554 (1990).

It is preferred that the expression vectors and the inserts which code for the single chain polypeptides have compatible restriction sites at the insertion junctions and that those

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restriction sites are unique to the areas of insertion. Both vector and insert are treated with restriction endonucleases and then ligated by any of a variety of methods such as those described in Sambrook et al., *supra*.

5   Preferred genetic constructions of vectors for production of dimers and multimers of the present invention are those which contain a constitutively active transcriptional promoter, a region encoding signal peptide which will direct synthesis/secretion of the nascent single chain polypeptide out of the cytoplasm into either an intracellular compartment (in eukaryotes) or the cell exterior (or periplasm in prokaryotes). Preferably, the expression rate is commensurate with the transport, folding and assembly steps to avoid accumulation of the

10   polypeptide as insoluble material. In addition to the replicon and control sequences, additional elements may also be needed for optimal synthesis of single chain polypeptide. These elements may include splice signals, as well as transcription promoter, enhancers, and termination signals.

Vectors which are commercially available can easily be altered to meet the above

15   criteria for a vector. Such alterations are easily performed by those of ordinary skill in the art in light of the available literature and the teachings herein.

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host microorganism. Such a drug resistance or other selectable marker is intended in

20   part to facilitate in the selection of transformants. Additionally, the presence of a selectable marker, such as a drug resistance marker, may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, such a pure culture of the transformed host microorganisms would be obtained by culturing the microorganisms under conditions which require the induced phenotype for survival.

25   Recovery and purification of the dimers and multimers of the present invention can be accomplished using standard techniques known in the art. For example, if the polypeptides are excreted into the culture medium, the polypeptides can be concentrated by ultrafiltration and then separated from monomers by gel filtration. When the polypeptides are transported to the periplasmic space of a host cell, purification can be accomplished by

30   osmotically shocking the cells, and proceeding with ultrafiltration, antigen affinity column chromatography or column chromatography using an ion exchange chromatography and gel filtration. Polypeptides which are insoluble and present as refractile bodies, also called inclusion bodies, can be purified by lysis of the cells, repeated centrifugation and washing to isolate the inclusion bodies, solubilization, such as with guanidine-HCl, and then passage of the

35   solubilized material through a gel filtration column.

While not wishing to be bound by theory, it is believed the dimers and multimers of the present invention form as a result of two discrete physicochemical states (isoforms) of the single chain polypeptide. In one transition state (A) or folding intermediate, only

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monomers will be formed. In the s cond transition stat (B) only dimers, or multiples thereof will be formed. The transition states are believed to be related to a conformational or charge equilibrium at a single residue or region in either polypeptide of the single chain polypeptide or even in the linker joining the two. During synthesis, since the single chain polypeptide has

5   not yet assumed a final stable three dimensional structure, the single chain polypeptide is thought to be fairly random in configuration. Single chain polypeptides in this later transition state are capable of forming interchain dimers, a dimer being capable of non-covalently linking to another dimer or multimer.

The activity of the single chain polypeptides can be measured by standard assays

10   known in the art, for example competition assays, enzyme-linked immunosorbant assay (ELISA), and radioimmunoassay (RIA).

USES

The dimers and multimers of the present invention provide unique benefits for use in diagnostic and therapeutics, such as for use in diagnosis or therapy of a variety of

15   diseases. In particular, the dimers and multimers are useful for, but not limited to, *in vivo* and *in vitro* uses in diagnostics, therapy, imaging and biosensors.

Essentially all of the uses for which monoclonal or polyclonal antibodies, or fragments thereof, have been envisioned by the prior art, can be addressed by the multivalent proteins of the present invention. These uses include detectably-labelled forms of the

20   multivalent protein. Types of labels are well-known to those of ordinary skill in the art. They include radiolabelling, chemiluminescent labeling, fluorochromic labelling, and chromophoric labeling. Other uses include imaging the internal structure of an animal (including a human) by administering an effective amount of a labelled form of the multivalent protein and measuring detectable radiation associated with the animal. They also include improved

25   immunoassays, including sandwich immunoassay, competitive immunoassay, and other immunoassays wherein the labelled antibody can be replaced by the multivalent antigen-binding protein of this invention.

The dimers and multimers may be incorporated into a pharmaceutically acceptable carrier. Injectable compositions of the present invention may be either in

30   suspension or solution form. In solution form the complex (or when desired the separate components) is dissolved in a pharmaceutically acceptable carrier. Such carriers comprise a suitable solvent, preservatives such as benzyl alcohol, if needed, and buffers. Useful solvents include, for example, water, aqueous alcohols, glycols, and phosphonate or carbonate esters. Such aqueous solutions generally contain no more than 50 percent of the organic solvent by

35   volume.

Dimers and multimers composed of antigen specific antibody domains are particularly advantageous for use in the diagnosis and/or therapy of diseases, such as cancer, where target antigens are often expressed on the surface of cells. For diagnostic and/or

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therapeutic uses, the dimers or multimers can be conjugated with an appropriate imaging or therapeutic agent. Examples of antibodies from which the V_L and V_H domains can be obtained to produce dimers or multimers are CC antibodies, such as CC49, disclosed in published PCT Application WO 89/00692 on January 26, 1989 and published PCT Application WO 90/04410 on May 3, 1990.

Methods for preparing and administering conjugates of the dimers and multimers are accomplished by methods well known or readily determined, as described, for example, in Goldenberg et al., *New England J. Med.*, 298, 1384-1388 (1978); Goldenberg et al.,*Gastroenterol.*, 84, 524-532 (1983); Siccardi et al., *Cancer Res.*, 46, 4817-4822 (1986); Keenan et al., *J. Nucl. Med.*, 25, 1197-1203 (1984); Meares et al., *Anal. Biochem.*, 142, 68-78 (1984). Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Conjugates of the dimers and multimers and an imaging marker are administered in a pharmaceutically effective amount for the *in vivo* diagnostic assays, and then detecting the presence of the imaging marker by appropriate detection means. Generally, the dosage should be effective to visualize or detect tumor sites, distinct from normal tissues. Preferably, a one-time dosage will be between 0.1 mg to 200 mg of the conjugate of the dimer or multimer and imaging marker per patient.

Examples of imaging markers which can be conjugated to the dimers and multimers are well known and include substances which can be detected by diagnostic imaging using a gamma scanner or hand held gamma probe, and substances which can be detected by nuclear magnetic resonance imaging using a nuclear magnetic resonance spectrometer

Suitable but not limiting examples of substances which can be detected using a gamma scanner include [125I], [131I], [123I], [111In], [105Rh], [153Sm], [67Cu], [67Ga], [166Ho], [177Lu], [186Re], [188Re] and [99mTc]. An example of a substance which can be detected using a nuclear magnetic resonance spectrometer is gadolinium.

A pharmaceutically effective amount of a composition containing dimers and multimers of antibodies is that which should be sufficient to achieve effective binding with the antigens against which the antibodies have specific affinity.

Examples of antibody-therapeutic agent conjugates which can be used in therapy include dimers or multimers of antibodies coupled to radionuclides, such as [131I], [90Y], [105Rh], [47Sc], [67Cu], [212Bi], [211At], [67Ga], [125I], [186Re], [188Re], [177Lu], [99mTc], [153Sm], [123I] and [111In]; to drugs such as methotrexate, adriamycin; to biological response modifiers, such as interferon and to toxins, such as ricin.

Methods of preparing and administering conjugates of the dimers and multimers of antibodies and a therapeutic agent are well known or readily determined. The pharmaceutical composition may be administered in a single dose or multipl dosage form.

Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Dimers and multimers containing single chain antibodies are particularly suitable for radioimmunoguided surgery (RIGS). In RIGS, an antibody labeled with an imaging marker is

5   injected into a patient having a tumor that expresses a target antigen. The antibody localizes to the tumor and is detected by a hand-held gamma detecting probe (GDP). The tumor is then excised (see, Martin et al. *Amer. J. Surg.*, 156, 386-392 (1988); Martin et al., *Hybridoma*, 5, S97-S108). An exemplary GDP is the Neoprobe™ scanner, commercially available from Neoprobe Corporation, Columbus, OH.

10   The invention will be further clarified by a consideration of the following examples, which are intended to be purely exemplary of the present invention.

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ABBREVIATIONS

	BCIP	5-bromo-4-chloro-3-indoyl phosphate
	bp	base pair
5	Bis-Tris propane	(1,3-bis[tris(hydroxymethyl)-methylamino]-propane)
	BSA	bovine serum albumin
	CDR	Complementarity determining region
	ELISA	enzyme linked immunosorbent assay
	Fv2	non-covalent single chain Fv dimer
10	IEF	isoelectric focusing
	Kbp	kilo base pair
	LB	Luria-Bertani medium
	Mab	monoclonal antibody
	MES	2-(N-Morpholino)ethane sulfonic acid
15	MW	molecular weight
	NBT	nitro blue tetrazolium chloride
	PAG	polyacrylamide gel
	PAGE	polyacrylamide gel electrophoresis
	PBS	phosphate buffered saline
20	PCR	polymerase chain reaction
	pSCFV	plasmid containing DNA sequence coding for SCFV
	RIGS	radioimmunoguided surgery
	RIT	radioimmunotherapy
25	scFv	single chain Fv immunoglobulin fragment
	SDS	sodium dodecyl sulfate
	TBS	Tris-buffered saline
	Tris	(Tris[hydroxymethyl]aminomethane)
	TTBS	Tween-20 wash solution
30	V_H	immunoglobulin heavy chain variable domain
	V_L	immunoglobulin light chain variable domain

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Antibodies

CC49: A murine monoclonal antibody specific to the human tumor-associated glycoprotein 72 (TAG-72) deposited as ATCC No. HB9459.

CC49 FAB: An antigen binding portion of CC49 consisting of an intact light chain linked to the N-terminal portion of the heavy chain.

ChCC49: Chimeric CC49 has the murine constant regions of the CC49 antibody replaced with the corresponding human constant regions.

CC49 scFv(X): Single chain antibody fragment consisting of two variable domains of CC49 antibody joined by a peptide linker. X refers to the theoretical pI of the scFv (e.g., CC49 scFv8.1).

CC49 Fv2: Two CC49 scFv non-covalently linked to form a dimer. The number after Fv refers to the number of monomer subunits of a given molecule, e.g., CC49 Fv6 refers to the hexamer multimers.

Hum4 V_L or H4VL: Variable domain of the human Subgroup IV kappa light chain.

SCFV UHM(X): SCFV consisting of a CC49 variable light chain and a CC49 variable heavy chain joined by a 25 amino acid linker named UNIHOPE. (X) refers to the theoretical pI of the SCFV which changes as amino acids are added to the C terminus, e.g., scFv UHM8.1; scFv UHM5.2 and scFv UHM5.2.

SCFV UHH: SCFV consisting of a Hum4 V_L and a CC49 variable heavy chain joined by a 25 amino acid linker named UNIHOPE.

EXAMPLES

General Experimental

Procedures for molecular cloning are as those described in Sambrook et al., *Molecular Cloning*, Cold Spring Harbor Press, New York, 2nd Ed. (1989) and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York (1992).

All water used throughout was deionized distilled water

Oligonucleotide Synthesis and Purification

All oligonuclotides (oligos) were synthesized on either a Model 380A or a Model 391 DNA Synthesizer from Applied Biosystems (Foster City, CA) using standard β-cyanoethyl phosphoramidites and synthesis columns. Protecting groups on the product were removed by heating in concentrated ammonium hydroxide at 55°C for 6 to 15 hours. The ammonium hydroxide was removed through evaporation and the crude mixtures were resuspended in 30 to 40 μL of sterile water. After electrophoresis on polyacrylamide-urea gels, the oligos were visualized using short wavelength ultraviolet (UV) light. DNA bands were excised from the gel and eluted into 1 mL of 100 mM Tris-HCl, pH 7.4, 500 mM NaCl, 5 mM EDTA over 2 hours at 65°C. Final purification was achieved by applying the DNA to Sep-Pac™ C-18 columns (Millipore, Bedford, MA) and eluting the bound oligos with 60 percent methanol. The

solution volume was reduced to approximately 50 µL and the DNA concentration was
determined by measuring the optical density at 260 nm (OD_{260}).

Bacterial Strains

5   Both *Eschericia coli* (*E. coli*) RR1 and *E. coli* GM 161 (dam⁻), containing the plasmid
pRW83, were obtained from Dr. J. O. Lampen (Rutgers University, New Brunswick, NJ)
E. coli DH1 was purchased from Invitrogen (San Diego, CA) and competent *E. coli* AG1 was
purchased from Stratagene (La Jolla, CA). The plasmid pCGS515 was obtained from
Collaborative Research, Inc. (Bedford, MA).

Restriction Enzyme Digests

10   All restriction enzyme digests were performed using Bethesda Research
Laboratories (Gaithersburg, MD), or New England Biolabs, Inc. (Beverly, MA) enzymes and
buffers following the manufacturer's recommended procedures. Digested products were
separated by polyacrylamide gel electrophoresis (PAGE). The gels were stained with ethidium
bromide, the DNA bands were visualized using long wavelength UV light and the DNA bands

15   were then excised. The gel slices were placed in dialysis tubing (Union Carbide Corp., Chicago)
containing 5 mM Tris, 2.5 mM acetic acid, 1 mM EDTA, pH 8.0 and eluted using a Max
Submarine electrophoresis apparatus (Hoefer Scientific Instruments, CA). Sample volumes
were reduced on a Speed Vac Concentrator (Savant Instruments, Inc., NY). The DNA was
ethanol precipitated and redissolved in sterile water.

20   Enzyme Linked Immunosorbent Assay (ELISA)

          TAG-72 antigen, prepared substantially as described by Johnson et al, *Can. Res.*,
46, 850-857 (1986), was adsorbed onto the wells of a polyvinyl chloride 96 well microtiter plate
(Dynatech Laboratories, Inc., Chantilly, VA) by drying overnight. The plate was blocked with
1 percent BSA in PBS for 1 hour at 31°C and then washed 3 times with 200 µL of PBS,

25   0.05 percent Tween-20. 25 µL of test antibodies and 25 µL of biotinylated CC49 (1/20,000
dilution of a 1 mg/mL solution) were added to the wells and the plate incubated for 30 minutes
at 31°C. The relative amounts of TAG-72 bound to the plate, biotinylated CC49, streptavidin-
alkaline phosphatase, and color development times were determined empirically in order not
to have excess of either antigen or biotinylated CC49, yet have enough signal to detect

30   competition by scFv. Positive controls were CC49 at 5 µg/mL and CC49 Fab at 10 µg/mL.
Negative controls were 1 percent BSA in PBS and/or concentrated LB. Unbound proteins were
washed away. 50 µL of a 1:1000 dilution of streptavidin conjugated with alkaline phosphatase
(Southern Biotechnology Associates, Inc., Birmingham, AL) were added and the plate was
incubated for 30 minutes at 31°C. The plate was washed 3 more times. 50 µL of a

35   para-nitrophenyl-phosphate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD)
were added and the color reaction was allowed to develop for a minimum of 20 minutes. The
relative amount f scFv binding was measured by optical density scanning at 404-450 nm using
a microplate reader (Molecular Devices Corporation, Manlo Park, CA). Binding of the scFv

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resulted in decreased binding of the biotinylated CC49 with a concomitant decrease in color development.

Amino-Terminal Sequence Analysis

5   Amino terminal amino acid sequencing was performed by the Edman degradation procedure using an Applied Biosystems model 470A Protein Sequencer (Foster City, CA). Fractions were analyzed with an interface to an Applied Biosystems Model 120A Analyzer (Hewrick, M.W. et al.). Protein samples (about 10 μg each) were prepared by removing PBS present in the sample by 2 cycles of water dilution and concentration using a Centrican 30 device (Amicon).

10   SDS-PAGE and Western Blotting

Samples for SDS-PAGE analysis (20 μL) were prepared by boiling in a non-reducing sample preparation buffer-Seprasol I (Integrated Separation Systems (ISS), Natick, MA) for 5 minutes and loaded on 10-20 percent gradient polyacrylamide Daiichi Minigels as per the manufacturer's directions (ISS).

15   Electrophoresis was conducted using a Mini 2-gel apparatus (ISS) at 55 mA per gel at constant current for approximately 75 minutes. Gels were stained in Coomassie Brilliant Blue R-250 (Bio-Rad, Richmond, CA) for at least 1 hour and destained. Molecular weight standards were prestained (Mid Range Kit, Diversified Biotech, Newton Center, MA) and included the following proteins: Phosphorylase b, glutamate dehydrogenase, ovalbumin, lactate

20   dehydrogenase, carbonic amhydrase, B-lactoglobulin and cytochrome C. The corresponding MWs are: 95,500, 55,000, 43,000, 36,000, 29,000, 18,400, and 12,400, respectively.

When Western analyses were conducted, a duplicate gel was also run. After electrophoresis, one of the gels was equilibrated for 15-20 minutes in anode buffer #1 (0.3 M Tris-HCl pH 10.4). An Immobilon-P PVDF (polyvinylidene dichlorine) membrane (Millipone,

25   Bedford, MA) was treated with methanol for 2 seconds, and immersed in water for 2 minutes. The membrane was then equilibrated in anode buffer #1 for 3 minutes. A Milliblot-SDE apparatus (Millipore) was utilized to transfer proteins in the gel to the membrane. A drop of anode buffer #1 was placed in the middle of the anode electrode surface. A sheet of Whatman 3MM filter paper was soaked in anode buffer #1 and smoothly placed on the electrode surface.

30   Another filter paper soaked in anode buffer #2 (25 mM tris pH 10.4) was placed on top of the first one. A sandwich was made by next adding the wetted PVDF membrane, placing the equilibrated gel on top of this and finally adding a sheet of filter paper soaked in cathode buffer (25mM Tris-HCl, pH 9.4 in 40 mM glycine). The screws for the cathode were firmly secured. Transfer was accomplished in 30 minutes using 250 mA constant current (initial

35   voltage ranged from 8-20 volts).

After blotting, the membrane was rinsed briefly in water and placed in a dish with 20 mL blocking solution (1 percent bovine serum albumin (BSA) (Sigma, St. Louis, MO) in Tris-buffered saline (TBS)). TBS was purchased from Pierce Chemical (Rockford, IL) as a

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pr weighed powder such that when 500 mL water is added, the mixture gives a 25 mM Tris, 0.15 M sodium chloride solution at pH 7.6. The membranes were blocked for a minimum of 1 hour at ambient temperature and then washed 3 times for 5 minut s each using 20 mL 0.5 percent Tween-20 wash solution (TTBS). To prepare the TTBS, 0.5mL of Tween 20 (Sigma) was

5   mixed per liter of TBS. The probe antibody used was 20 mL biotinylated FAID14 solution (10 µg per 20 mL antibody buffer). Antibody buffer was made by adding 1 g BSA per 100 mL of TTBS. After probing for 30-60 minutes at ambient temperature, the membrane was washed 3 times with TTBS, as above.

Next, the membrane was incubated for 30-60 minutes at ambient temperature

10   with 20 mL of a 1:500 dilution in antibody buffer of streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). The wash step was again repeated after this, as above. Prior to the color reaction, membranes were washed for 2 minutes in an alkaline carbonate buffer (20 mL). This buffer is 0.1 M sodium bicarbonate, 1 mM MgCl2.H20, pH 9.8. To make up the substrate for alkaline phosphatase, nitroblue tetrazolium

15   (NBT) chloride (50 mg, Sigma) was dissolved in 70 percent dimethylformamide. 5-Bromo- -4-chloro-3-indoyl phosphate (BCIP) (25 mg, Sigma) was separately dissolved in 100 percent dimethylformamide. 5-Bromo-4-chloro-3-indoyl phosphate (BCIP) 25 mg, Sigma) was separately dissolved in 100 percent dimethylformamide. These solutions are also commercially available as a Western developing agent sold by Promega. These solutions were stored at 4°C

20   in the dark for up to 3 months. For color development, 120 µL of each were added to the alkaline solution above and allowed to react for 15 minutes before they were washed from the developed membranes with water.

Biotinylated FAID14

FAID14 is a murine anti-idiotypic antibody (IgG2a, K isotype) deposited as ATCC

25   No. CRL 10256 directed against CC49. FAID14 was purified using a Nygene Protein A affinity column (Yonkers, NY). The manufacturer's protocol was followed, except that 0.1 M sodium citrate, pH 3.0 was used as the elution buffer. Fractions were neutralized to pH ~7 using 1.0 M Tris-HCl pH 9.0. The biotinylation reaction was set up as follows. FAID14 (1 mg, 100 µL in water) was mixed with 100 µL of 0.1 M Na_2CO_3 pH 9.6. Biotinyl-ε-amino-caproic acid N-hydroxy

30   succinimide ester (Biotin-X-NHS) (Calbiochem, LaJolla, CA) (2.5 mg) was dissolved in 0.5 mL dimethylsulfoxide. Biotin-X-NHS solution (20 µL) was added to the FAID14 solution and allowed to react at 22°C for 4 h. Excess biotin and impurities were removed by gel filtration, using a Pharmacia Superose 12 HR10/30 column (Piscataway, NJ). At a flow rate of 0.8 mL/min, the biotinylated FAID14 emerged with a peak at 16.8 min. The fractions making up this peak

35   were pooled and stored at 4°C and used to detect the CC49 idiotype as determined by the CC49 V_L and V_H CDRs.

Isoelectric Focusing (IEF) and Western Analysis

Isoelectric points (pI's) were predicted using a computer program called PROTEIN-TITRATE, available through DNASTAR (Madison, WI). Based on amino acid composition with an input sequence, a MW value is given, in addition to the pI. Since Cys residues contribute to the charge, the count was adjusted to 0 for Cys, since they are all involved in disulfide bonds.

Experimentally, pI's were determined using Isogel agarose IEF plates, pH range 3-10 (FMC Bioproducts, Rockland, ME). A Biorad Bio-phoresis horizontal electrophoresis cell was used to run the IEF, following the directions of both manufacturers. The electrophoresis conditions were: 500 volts (limiting), at 20 mA current and 10 W of constant power. Focusing was complete in 90 min. IEF standards were purchased from Biorad; the kit included phycocyanin, β-lactoglobulin B, bovine carbonic anhydrase, human carbonic anhydrase, equine myoglobin, human hemoglobins A and C, 3 lentil lectins and cytochrome C, with pI values of 4.65, 5.10, 6.00, 6.50, 7.00, 7.10 and 7.50, 7.80, 8.00, and 8.20 and 9.60 respectively. Gels were stained and destained according to the directions provided by FMC.

For IEF Westerns, duplicate gels were electrophoresed. Focused proteins in the second gel were transferred to PVDF membranes, as above, using a Biorad Model 483 slab dryer hooked up to a dry ice trap and vacuum pump. A piece of filter paper wetted in water was placed on the drier surface, followed by a prepared PVDF membrane, and then by the gel itself, with its solid support facing up. The plastic cover of the drier was then placed on top of this "sandwich". The drier was operated under vacuum without heat for 20 min to transfer the proteins in the gel to the membrane. Following the transfer, the blotted membrane was blocked and subsequently probed with biotinylated FAID 14 as described above for SDS-PAGE.

Quantitation of CC49 Antibody Species

Purified CC49 antibodies were quantitated by measuring the absorbence of protein dilutions at 280 mm using matching 1.0 mL 1.0 cm pathlength quartz cuvettes (Hellma) and a Perkin-Elmer UV/VIS Spectrophotometer, Model 552A. Molar absorptivities (E_m) were determined for each antibody by using the following formula:

$$E_m = (number\ Trp) \times 5,500 + (number\ Tyr) \times 1,340 +$$
$$(number\ (Cys)2) \times 150 + (number\ Phe) \times 10$$

The values are based on information given by D. B. Wetlaufer, *Advances in Protein Chemistry*, 17, 375-378).

High Performance Liquid Chromatography

All high performance liquid chromatography (HPLC) was performed using an LKB HPLC system with titanium or teflon tubing throughout. The system consists of the Model 2150 HPLC pump, Model 2152 controller, UV CORD SII Model 2238 detection system set at an absorbance of 276 nm and the Model 2211 SuperRac fraction collector.

EXAMPLE 1   Formati n of Single Chain Antibody Dimers in *E. coli*

A. Preparation of pSCFV 31

A vector was prepared fr m plasmid pRW 83 containing a chloramph nicol resistance (Camr) gene for clone selection, and a *penP* gene with a *penP* promoter and

5   terminator (see Mezes et al. (1983), *J. Biol. Chem.*, 258, 11211-11218) and the *pelB* signal sequence [see Lei et al., *J. Bact.*, 169, 4379-4983 (1987)]. The vector designated Fragment A (see Figure 1) was prepared by removal of the *penP* gene with a *Hind* III/*Sal* I digest.

The *penP* promoter and *pelB* signal sequence were obtained by a PCR using pRW 83 as a template and oligonucleotides penP1 and penP2 as primers. The fragment was

10   designated Fragment B (see Figure 1). A *Nco* I enzyme restriction site was introduced at the 3' end of the signal sequence region by the penP2 oligonucleotide.

penP1:

5'-CGATAAGCTTGAATTCCATCACTTCC-3'

penP2:

15   5'-GGCCATGGCTGGTTGGGCAGCGAGTAATAACAATCCAGCG GCT GCCGTAGGCAATAGGTATTTCATCAAAATCGTCTCCCTCCGTTTGAA-3'

A scFv comprised of a Hum4 V_L, a CC49 V_H, and an 18 amino acid linker (Lys Glu Ser Gly Ser Val Ser Ser Glu Gln Leu Ala Gln Phe Arg Ser Leu Asp) was obtained from pCGS515/SCFV1 by PCR using oligonucleotides penP3 and penP6. This fragment was

20   designated Fragment D (see Figure 2). The complete nucleotide sequence and amino acid sequence of pCGS515/SCFV1 is given in Figure 3. A *Bcl* I site was introduced at the 3' end of the V_H region by the penP6 oligonucleotide.

penP3:

5'-GCTGCCCAACCAGCCATGGCCGACATCGTGATGACCCAGTCTCC-3'

25   penP6(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTG ACG GATTCATCGCAATGTTTTTATTTGCCGGAGACGGTGACTGAGGTTCC-3'

Fragments B and D were joined by SOE-PCR (splicing by overlap extention-polymerase chain reaction) using oligonucleotides penP1 and penP6, following the

30   procedures of Horton et al., *Gene*, 77, 61-68 (1989). The new fragment was designated E (See Figure 2).

Fragment C, containing the *penP* termination codon, was isolated by digesting pRW 83 with *Bcl* I and *Sal* I. pRW 83 was isolated from *E. coli* strain GM161, which is DNA methylase minus or *dam*.

35   Plasmid pSCFV 31 (see Figure 2) was created with a three part ligation of Fragments A, C, and E.

B. Preparation of pSCFV 31b

The *Nco* I restriction enzyme site within the Cam^r gene and the *Hind* III site located at the 5' end of the *penP* promoter in pSCFV 31 were destroyed through a PCR DNA amplification using oligonucleotides Nco1.1 and Nco1.3(-) to generate an *Eco* RI to *Nco* I

5   fragment and oligonucleotides Nco1.2 and Nco1.4c(-) to generate a *Nco* I to *Eco* RI fragment. These two fragments were joined by PCR-SOE using oligonucleotides Nco1.1 and Nco1.4c(-). The oligonucleotides are set forth below:

Nco1.1:

5'-TCCGGAATTCCGTATGGCAATGA-3'

10 Nco1.3(-):

5'-CTTGCGTATAATATTTGCCCATCGTGAAAACGGGGGC-3'

Nco1.2:

5'-ATGGGCAAATATTATACGCAAG-3'

Nco1.4c(-):

15 5'-CACTGAATTCATCGATGATAAGCTGTCAAACATGAG-3'

pSCFV 31 was digested with *Eco* RI and the larger fragment was isolated by polyacrylamide gel electrophoresis. To prevent self ligation, the DNA was dephosphorylated using calf intestinal alkaline phosphatase according to the teachings of Sambrook et al., *supra*.

A two part ligation of the larger pSCFV 31 digested fragment and the PCR-SOE

20 fragment, described above, resulted in the creation of pSCFV 31b (see Figure 4).

C. Preparation of pSCFV 33H

pSCFV 31b was digested with *Nco* I and *Sal* I and a fragment containing the Cam^r gene was isolated.

The Hum4 V_L was obtained by PCR DNA amplification using pCGS515/SCFV1 (See

25 Figure 3) as a template and oligonucleotides 104BH1 and 104BH2(-) as primers.

104BH1:

5'-CAGCCATGGCCGACATCGTGATGACCCAGTCTCCA-3'

104BH2(-):

5'-AAGCTTGCCCCATGCTGCTTTAACGTTAGTTTTATCTGCTGG

30 AGACAGAGTGCCTTCTGCCTCCACCTTGGTCCCTCCGCCGAAAG-3'

The CC49 V_H was obtained by PCR using p49γ1-2.3 (PCT WO 90/04410) as a template and oligonucleotides 104B3 and 104B4(-) as primers. A *Nhe* I enzyme restriction site was introduced just past the termination codon in the 3' end (before the *Bcl* I site) by oligonucleotide 104B4(-).

35 104B3:

5'-GTTAAAGCAGCATGGGGCAAGCTTATGACTCAGTTGCAGCAGTCTGACGC-3'

104B4(-):

.5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTGACGGATT
CATCGCTAGCTTTTTATTTGCCATAATAAGGGGAGACGGTGACTGAGGTTCC-3'

In the PCR which joined these two fragments using ligonucleotides 104BH1 and
104B4(-) as primers, a coding region for a 22 amino acid linker was formed.

5   A fragment C (same as above) containing the penP termination codon was
isolated from pRW 83 digested with Bcl I and Sal I.

Plasmid pSCFV 33H (see Figure 4) was created with a three part ligation of the
vector, fragment C, and the SCFV fragment described above.

D. Preparation of pSCFV UNIH

10   To create a vector for any SCFV with the desired restriction sites in place, a
plasmid designated UNIH was created by digesting pSCFV 33H with Ncol and Nhel, and
isolating the DNA fragment containing the Camr gene.

Hum4 V_L was obtained by PCR DNA amplification using pRL1001 as a template
and oligonucleotides UNIH1 and UNIH2(-) as primers. The DNA sequence in pRL1001 coding for
15 the Hum4 V_L is given in Figure 5. Oligonucleotides for the PCR were:
UNIH1:

5'-CAGCCATGGCCGACATTGTGATGTCACAGTCTCC-3'
The Nco I site is in bold and the hybridizing sequence is underlined.
UNIH2(-):

20 5'-GAGGTCCGTAAGATCTGCCTCGCTACCTAGCAAA
AGGTCCTCAAGCTTGATCACCACCTTGGTCCCTCCGC-3'
The Hind III site is in bold.

The CC49 V_H was obtained by a PCR using p49γ1-2.3 as a template and
oligonucleotides UNI3 and UNI4(-) as primers.
25 UNI3:

5'-AGCGAGGCAGATCTTACGGACCTCGAGGTTCAGTTGCAGCAGTCTGAC-3'.
The Xho I site is in bold and the hybridizing sequence is underlined.
UNI4(-):

5'-CATCGCTAGCTTTTTATGAGGAGACGGTGACTGAGGTTCC-3'.
30 The Nhe I site is in bold and the hybridizing sequence is underlined.

Oligonucleotides UNIH1 and UNI4(-) were used in the PCR-SOE amplification
which joined the Hum4 V_L and CC49 VH fragments and formed a coding region for a negatively
charged fifteen amino acid linker. The DNA was digested with Nhe I and Nco I and ligated with
the vector fragment from the Nco I-Nhe I digest of pSCFV 33H. The resultant plasmid was
35 designated pSCFV UNIH (see Figure 4).

With the construction of pSCFV UNIH, a universal vector for any SCFV was created
with all the desired restriction enzyme sites in place.

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E. Preparation of pSCFV UHH and pSCFV UHM

pSCFV UNIH was digested with Hind III/Xho I, and the large DNA fragment containing the Cam^r gene, Hum4 V_L and CC49 V_H was isolated.

A fragment coding for a 25 amino acid linker, UNIHOPE, was made by annealing the two oligonucleotides shown below. Annealing conditions were as follows: 10 μg of each oligo were added to Bethesda Research Laboratories (Gaithersburg, MD) enzyme Buffer #2 for a total volume of 53 μL, the mixture was heated to 98°C for one minute, cooled slowly to 40°C and placed at 4°C overnight. Following digestion with Hind III/Xho I, the linker was electrophoresed through a 6 percent PAG. The DNA bands were excised from the gel and the DNA was electroeluted for five minutes at 200 volts. The DNA was pelleted by ethanol precipitation and dissolved in water. The linker UNIHOPE is based on 205C SCA™ linker (see Whitlow et al., Methods: A Companion to Methods in Enzymology, 2, 97-105 (1991), but the first amino acid was changed from serine to leucine and the twenty-fifth amino acid were changed from glycine to leucine, to accommodate the Hind III and Xho I restriction sites, respectively. The nucleotide sequence encoding the linker UNIHOPE is set forth below:

UNIHOPE (Top Strand):

5'-TATAAAGCTTAGTGCGGACGATGCGAAAAAGGATGCTGCGAAG
AAGGATGACGCTAAGAAAGACGATGCTAAAAAGGACCTCGAGTCTA-3'

UNIHOPE(-) Bottom Strand:

5'TAGACTCGAGGTCCTTTTTAGCATCGTCTTTCTTAGCGT CAT
CCTTCTTCGCAGCATCCTTTTTCGCATCGTCCGCACTAAGCTTTATA-3'

The resulting annealed strand was digested with Hind III/Xho I and ligated into the vector, thus generating the plasmid pSCFV UHH (shown in Figure 6). Plasmid pSCFV UHH expresses a biologically active, TAG-72 binding SCFV consisting of the Hum4 V_L and CC49 V_H. The expression plasmid utilizes the β-lactamase penP promoter, pectate lyase pelB signal sequence and the penP terminator region. Different immunoglobulin light chain variable regions can be inserted in the Nco I-Hind III restriction sites, different SCFV linkers can be inserted in the Hind III-Xho I sites and different immunoglobulin heavy chain variable regions can be inserted in the Xho I Nhe I sites.

F. Preparation of pSCFV UNIM and pSCFV UHM

pSCFV UNIM was created in conjunction with pSCFV UNIH. It differs in that the DNA sequence coding for the CC49V_L is incorporated into UNIM as opposed to the DNA sequence coding for the Hum4 V_L. The sequence for murine CC49V_L is disclosed in PCT WO 90/04410, published May 3, 1990, and was produced by PCR using the oligos UNIM1 and UNIM2(-) as primers. The CC49 V_H and the vector fragment were obtained by the manner described above for pSCFV UNIH. The SCFV insert was generated by PCR SOE using oligos UNIM1 and UNI4(-) to join the light and heavy chains and form a coding region for a negatively

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charged fifteen amino acid linker. Following *Nhe*I and *Nco*I digestion and purification of the

vector and insert, a ligation of the two pieces produced pSCFV UNIM.

UNIM1:

5'CAGCCATGGCCGACATTGTGATGTCACAGTCTCC-3'

5 UNIM2(-):

5'GAGGTCCGTAAGATCTGCCTCGCTACCTAGCAAAA

   GGTCCTCAAGCTTCAGCACCAGCTTGGTCCCAGCAC-3

          pSCFV UHM was generated by digesting pSCFV UNIM with *Hind*III and *Xho*I,

isolating the vector fragment with the Cam^r gene by 4 percent PAG, and ligating the

10 twenty-five amino acid linker, UNIHOPE, into the vector DNA fragment piece. The procedure

was the same as described for pSCFV UHH. pSCFV UHM contains DNA sequence coding for an

scFv having the CC49 V$_L$ and CC49 V$_H$ with a computer predicted pI of 8.1. Hence, this plasmid

was named pSCFV UHM8.1. Hence, this plasmid was named pSCFV UHM8.1

          The plasmid map of pSCFV UHH and pSCFV UHM are illustrated in Figure 6A. For

15 pSCFV UHH, V$_L$ is Hum4 V$_L$, the linker (L) is UNIHOPE and V$_H$ is CC49 V$_H$. For the pSCFV UHM

series, V$_L$ is CC49 V$_H$, the linker is UNIHOPE and V$_H$ is CC49V$_H$. For the pSCFV UHM(X) series, X

indicates the pI of the monomer product (pI = 8.1, 5.8 or 5.2) as described herein. Figures 6B

and 6C illustrate the monomeric (scFv) and dimeric (Fv2) forms, respectively, of the products.

The CDR regions are indicated schematically by the black bands.

20   The DNA and amino acid sequences of the scFv obtained from pSCFV UHH

constructions are given in Figure 7. The UNIHOPE linker sequence is indicated in bold and

underlined, while the CDR regions are in bold and italicized. The amino terminus of the

mature scFv product is indicated by + 1.

          E. coli AG1 (Statagene, La Jolla, CA) cells were transformed with pSCFV UHH and

25 pSCFV UHM8.1 by following the manufacturer's instructions. Competent cells were thawed on

ice and gently mixed by hand before 50 µL aliquots were dispensed into 15 mL polypropylene

tubes. A fresh dilution of β-mercaptoethanol was added to the tubes for a final concentration

of 25 mM. The cells were swirled gently every two minutes for ten minutes. Either 1 µL or 5 µL

of the prepared DNA was added to the tubes. The cells and DNA sat on ice for 30 minutes, were

30 heat pulsed in a 42°C water bath for 45 seconds and put back on ice for two minutes. SOC

medium (450 µL) (medium containing bacto-tryptone, bacto-yeast extract, NaCl MgCl$_2$ and

glucose) was added to each tube and the tubes were incubated at 37°C for one hour with

shaking. Either 100 µL or 200 µL of transformed cells were plated on LB agar containing

20 µg/ml chloramphenicol. Plates were incubated at 37°C overnight.

35   From each transformation, twelve to eighteen bacterial colonies were selected

for overnight growth at 37°C in 2 mL of LB/CAM 20. A portion of the overnight culture (1.2 mL)

was used for a small scale plasmid preparation following the boiling method adapted from

Holmes and Quigley (Sambrook et al, 1989, *supra*). Plasmid DNA was digested with restriction

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enzymes and the resultant fragments were size analyzed by agarose gel electrophoresis using ethidium bromide and long wavelength UV for visualization. The remaining 0.8 mL of overnight culture was sonicated (Soniprep 150, MSE Ltd., Sussex), then microcentrifuged for 3 minutes to pellet the debris. The supernatant was reduced in volume by 50 to 80 percent

5   using a Centricon 10 microconcentrator device (Amicon, Beverly, MA) and tested for biological activity against TAG-72.

(G) Construction of pSCFV UHM5.8 and pSCFV UHM5.2

Two Plasmids, pSCFV UHM5.8 and pSCFV UHM5.2 were constructed to express CC49 scFvs and CC49 Fv2s having computer predicted pI's of 5.8 and 5.2, respectively. Extra

10   negatively charged amino acids were added to the carboxyl terminus of the CC49 V_H, by synthesizing two oligonucleotides (oligos) and using them for a polymerase chain reaction (PCR) amplification of the CC49 V_L - linker - CC49 V_H DNA present in pSCFV UHM8.1. These oligos for the 3' end of the target are as follows. For the 5.8 construction, a 58-mercalled SCUHPM 5.8 was made. For the 5.2 construction, a 64-mercalled SCUHPM 5.2 was made.

15   SCUHPM 5.8

5'-AACA GCTAGC TTT TTA GGA GTC ATA GTC CTC AGG GGA GAC GGT GAC TGA GGT TCC TTG-3'

SCUHPM 5.2

5' - AACA GCTAGC TTT `TA CTC ATA CTC TTC AGG GTC TTC AGG GGA GAC GGT GAC TGA GGT

20   TCC TTG - 3'

The nucleotides representing Nhe I restriction sites are indicated in bold. These oligos were purified from a 14 percent polyacrylamide gel containing urea (Sambrook et al., supra). The 5' oligo for the PCR was a 34 mer designated UNIM1 (sequence given above).

The nucleotides representing an Nco I restriction site are indicated in bold. For

25   the PCR's, 100 pmol of the 5'(UNIM1) and 3' (SCUHPM 5.8) oligos were used to generate the CC49 scFv 5.8 DNA product insert, while 100 pmol of each of UNIM1 and SCUHPM 5.2 were used to generate the CC49 scFv 5.2 DNA product insert. The target DNA for the oligos in the PCR reaction (100 uL) was 1.0 ng of pSCFV UHM8.1. The reaction was performed using a programmable thermal controller (MJ Research Inc., Watertown, MA) and Taq polymerase

30   according to the directions provided by Perkin Elmer Cetus (Norwalk, CT). There were 30 cycles consisting of 40 seconds at 94°C, 70 seconds at 45°C and 75 seconds at 72°C. For the final cycle, the temperature was held at 72°C for 2 minutes. The PCR products were analyzed on a 1.0 percent agarose gel containing ethidium bromide and found to be the expected sizes of 788 bp and 794 bp for the 5.8 and 5.2 CC49 scFv constructions, respectively. The DNA was

35   preparatively purified from a 4 percent polyacrylamide gel, digested with Nco I (from BRL, Gaithersburg, MD) and Nhe I (from New England Biolabs) using New England BioLabs 10 x buffer #2 (10mM Tris-Hcl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM OTT, pH 7.9).

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The resulting trimmed *Nco* I and *Nhe* I oligonucleotides fragment of the correct size were purified away from the DNA inserts by electrophoresis in a 4 percent polyacrylamide gel. The CC49 scFv inserts were electroeluted and ethanol precipitated (Sambrook). The vector for ligation with these inserts had been prepared from pSCFV33H digested with *Nco* I and

5   *Nhe* I. Competent *E. coli* AG1 cells (Strategene, La Jolla, CA) were transformed with the ligated DNA and plated on LB plates containing 20 μg/mL chloramphenicol.

Four clones were chosen from each transformation to prepare miniprep plasmid DNA (Sambrook) for restriction enzyme analysis. Based on these results single clones were chosen; *E. coli* AG1/pSCFV UHM5.8 *E. coli* and AG1/pSCFV UHM5.2.

10 The DNA and amino acid sequences of the CC49 species, pSCFV UHM8.1 (A), 5.8 (B) and 5.2 (C), are given in Figure 8.

Preparation of Total Cellular Protein and *Escherichia Coli* Periplasimic Shockates

For analysis of total cell CC49 scFv activity or preparation of samples for sodium dodecyl sulfate polyacrylamide gel electrophonesis (SDS-PAGE), *E. coli* (pSCFV UHM8.1; pSCFV

15 5.8; or pSCFV 5.2) liquid cultures (2.5 mL LB supplemented with 20 μg/mL chloramphenicol) were grown overnight at 37°C. Cells from 1.5 mL of culture were pelleted in a microcentrifuge, the supernatant discarded, and the cell pellets resuspended in 0.5 mL phosphate buffered saline (PBS) (Sigma Chemical). Sample tubes were placed in an ethanol ice bath before sonication (MSE Soniprep 150, UK) using 3 cycles of disruption at 14 microns amplitude for 15

20 seconds each and cool down for 45 seconds in between. The sonicated cell debris was pelleted in a microcentrifuge, and the supernatant, representing the total, soluble protein fraction, was filtered through a Millex-GV 0.22 μ filter disc (Millipore, Bedford, MA).

For purification of the CC49 scFv[(X)] derivatives, where x = 8.1, 5.8 and 5.2, *E. coli* periplamsic fractions were prepared from 1.0 L overnight cultures. A 1.0 L culture was divided

25 into 4x250 mL portions and centrifuged at 5,000 rpm, for 10 minutes in a Sorvall GS-3 rotor. The pelleted cells were washed and resuspended in 100 mL each of 10 mM Tris-HCl pH 7.3 containing 30 mM NaCl. The cells were again pelleted at 5,000 rpm for 10 minutes. Cells were washed with a total of 100 mL of 30 mM Tris-HCl (pH 7.3) and pooled into 1 tube. To this was added 100 mL of 30 mM Tris-HCl pH 7.3 containing 40 percent w/v sucrose and 2.0 mL of 10 mM

30 EDTA, pH 7.5. The mixture was kept at ambient temperature, with occasional shaking for 10 minutes. The hypertonic cells were centrifuged as above. The cells where then quickly resuspended in ice cold 0.5 mM MgCl$_2$ and the suspension kept on ice for 10 minutes with occasional shaking. The cells were centrifuged as above and the shockate, containing the *E. coli* periplasmic fraction was further clarified by passing through a 0.2 μ filter apparatus

35 (Nalge Co., Rochester, NY). This material was concentrated using Centriprep 30 and Centricon 30 ultrafiltration devices (Amicon Beverly, MA) to a final volume of 1-2 mL.

For isolation and analysis of just cytoplasmic proteins, previously shocked *E. coli* cells (0.5-1.0 mg - wet weight) were resuspended in 0.5 mL PBS and sonicated as above. The cytosol was recovered by microcentri-fugation and filtered through a 0.2 μ filter device.

Prior to further purification, the shockate was dialyzed against 50 mM sodium acetate buffer, pH 4.95, using a System 500 microdialyzer unit (Pierce Chemical, Rockford, IL), with 3-4 exchange of buffer. An 8,000 molecular weight (MW) cut-off membrane was used.

Purification of CC49 Single Chain Monomer and Dimer Species

The general purification of the single chain antibody species is given in Scheme 1. The procedure under column 1 was initially used for purification of antibodies from pSCFV UHM8.1 and was later modified as given under column 2. Single chain antibody species derived from pSCFV UHM5.2 were purified as given under column 3. Final purification of the pSCFV UHM5.8 products was not completed.

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Periplasmic Fraction From		
1.	**2.**	**3.**
<u>E. coli</u> pSCFV UHM8.1 ↓ Mono S 50 mM NaOAC, pH 4.95 (Figure 9) ↓ 2 Pools of Activity Take Pool 2 ↓ Mono Q 20 mM Tris-HCl, pH 7.60 (Figure 10) ↓	<u>E. coli</u> pSCFV UHM8.1 ↓ Mono S 50 mM NaOAc, pH 5.20 (Gave smaller pellet upon dialysis) All active fractions pooled ↓ Mono Q 20 mM Tris-HCL, pH 7.60 ↓ Mono S 50 mM MES, pH 5.60 ↓	<u>E. coli</u> pSCFV UHM5.2 ↓ Superdex 75 Take monomer fractions (Figure 10) ↓ Mono Q 20 mM Bis-Tris Propane-HCl, pH 6.80 (Figure 11) ↓
Superdex 75 Result: pure dimer partially pure monomer (Figure 11)	Superdex 75 Result: (a) Pure dimer - Elutes at 43 min. using PBS buffer and 0.2 ml/min flow rate. (b) pure monomer-elutes at 52 min. using PBS and 0.2 mL/min. flow rate.	Superdex 75 Result - pure scFv. Elutes at 49.5 min. using PBS and 0.2 mL/min flow rate. (Figure 12)

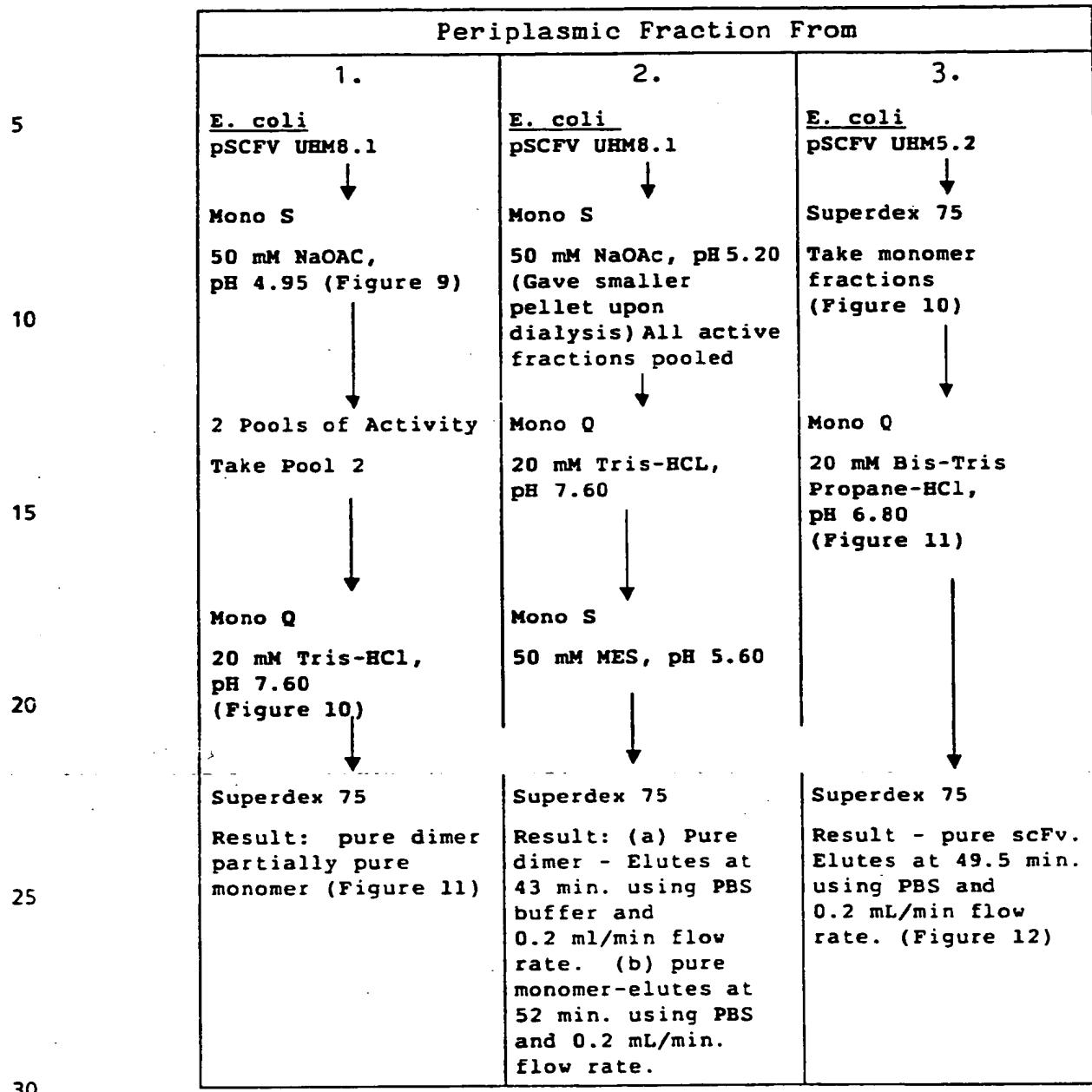-26-

A. <u>CC49 scFv 8.1</u>

For single chain antibody species from pSCFV UHM8.1, about half of the *E. coli* AG1/pSCFV UHM8.1 periplasmic shockate prepared from 1 L of culture as described above was dialyzed overnight against 50 mM sodium acetate buffer, pH 4.95. Precipitated proteins were

5   pelleted by microcentrifugation and the supernatant was applied to a cation exchange column (Mono S HR5/5 HPLC column, Pharmacia, Piscataway, N.J.). A linear gradient program utilizing 50 mM sodium acetate pH 4.95 as buffer A and 50 mM sodium acetate pH 4.95 containing 0.3 M NaCl as buffer B was run as follows:.

10

Time (min)	Flow Rate (mL/min)	Percent B
0	1.5	0
5.0	1.5	0
35.0	1.5	30
40.0	1.5	50
45.0	1.5	100
50.0	1.5	100
55.0	1.5	0

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When the periplasmic fraction was chromatographed over Mono S, two areas of activity were observed (Figure 9) and the two pools kept separate for the next step. The lanes 1-9 for SDS-PAGE correspond to a crude periplasmic fraction (lane 1) and fractions 1, 2, 3, 12, 16,

25   21, 26 and 43, lanes 2-9 respectively, with a Western transfer of the samples from a duplicate gel, indicate product in fractions 26 and 43 (lanes 17 and 18).

Pool 2 from the Mono S purification was concentrated to below 250 μL using a Centricon 30 device and dialyzed against buffer A (20 mM Tris-HCl, pH 7.60) for rechromatography on the Mono Q HR5/5 anion exchange column (Pharmacia, Piscataway, N.J.).

30   Buffer B was the same as buffer A, but in 0.5 M NaCl. The gradient program used was the same as for the Mono S run above, but for this Mono Q run the Mono S pool 2 sample was changed to 100 percent B the gradient was at 16 percent B.

The anion exchange chromatography and SDS-PAGE of the Mono S Pool 2 sample is given in Figure 10. The arrow on the chromatogram indicates the point in the gradient of

35   Buffer B (16 percent) where the percent of B was changed to 100 and held for 5 minutes. Lanes 1-9 correspond to fractions 1-3 of a similar chromatography (not shown) done on Pool 1 (Figure 9); a sample of Pool 1; a sample of Pool 2; and fractions 1-4 from the anion exchange

chromatography of Pool 2. Lanes 10-18 represent the same fractions as in lanes 1-9 in a
Western transfer fr m a duplicate gel.

The final step in the purification utilized a Superdex 75 HR10/30 gel filtration
column (Pharmacia). Potassium phosphate buffer (0.175 M, pH 6.85) was used as the mobile

5   phase, at a flow rate of 0.2 mL/min. Fraction 1 from the Mono Q purification was applied to the
Superdex column and the resulting chromatogram is shown in Figure 11A-1. Both peaks
emerging at 42 and 50.5 minutes were active in the competition assay, with considerably more
activity being observed in the first peak (42 minutes). On SDS-PAGE and Western transfer
(Figure 11B) a band for the correct molecular weight of monomeric single chain antibody was

10   observed. Lanes B1 and 8 are ChCC49 Fab; lanes 2 and 9, fraction 14; lanes 3 and 10, fraction
15; lanes 4 and 11 fraction 16; lanes 5 and 12, fraction 17,; lanes 6 and 13, fraction 18; and
lanes 7 and 14, fraction 19. Application of a ChCC49 Fab fragment to the gel filtration column
(Figure 11 A-2), using the same conditions as for the scFv sample, indicated that the scFv peak
emerging at 42 minutes contained a protein that was larger than the Fab, which emerged at

15   45.8 minutes. When the peak emerging at 42 minutes was subjected to the reducing condition
of SDS-PAGE, a monomeric unit on the gel and Western was seen, indicating a con-covalently
linked dimeric (Fv2) form of the single chain antibody. Further evidence of a unique dimeric
entity was obtained when an IEF gel was run.

IEF of fractions 15 (lanes C1 and C3) and 17 (lanes C2 and C4), indicates there is a

20   distinct difference between the pI of the Fv2 (lanes 1 and 3) and the monomeric scFV (lanes 2
and 4). Samples in lanes 1 and 2 were stained with Coomassie Brilliant Blue R-250, while lanes 3
and 4 represent a Western transfer of the corresponding lanes of a duplicate IEF gel. The faint
FAID14 positive band in Figure 11C, lane 3, above the main Fv2 band is not known. It could be a
trace degradation product or some higher multimer.

25   A second purification system was then developed to optimize the purification of
the monomer, dimer and multimer species of the pI 8.2 single chain antibodies. The procedure
is outlined in column two of Scheme 1. The buffer system for purification with the Mono S
cation exchange column was also changed to 50 mM sodium acetate pH 5.2 and 50 mM
2-(N-morpholino)ethane sulfonic acid (MES).

30   The protocol which afforded pure CC49 scFv and Fv2 8.2 was as follows: A
periplasmic fraction was dialyzed and applied to mono S using a 50 mM sodium acetate buffer
system, as before, but at pH 5.20. At this pH less protein precipitated than at pH 4.95 but the
results were much the same in terms of the chromatography. Next, the Mono Q column was
utilized as above. After this, a second Mono S run was introduced for the active Mono Q

35   fractions. The buffer system used was: buffer A (50 mM MES, pH 5.6) and buffer B being the
same as buffer A in 0.5 M NaCl. The monomer and dimer were finally purified using the
Superdex 75 HR10/30 column and PBS as the buffer.

B. <u>CC49 scFv5.2 and Fv2-5.2</u>

The periplasmic shockate from a 1.0 liter culture was prepared as described above. The strategy for purification used here was to separate the monomer from the dimer in a gel filtration step first. A Superdex 75 prep grade HR 26/60 preparative column was used. The

5   concentrated crude sample (0.5 mL) was chromatographed at a flow rate of 1.3 mL/min. using 0.175 M potassium phosphate buffer pH 6.8. See Figure 12 for the chromatogram. The elution times for the molecular weight standards: (1) bovine IgG (Pharmacia), MW = 153 Kd; (2) chicken ovalbumin, MW = 44 Kd; and (3) horse myoglobin, MW 17 Kd from a separate run are as indicated by the arrows.

10   SDS-PAGE analysis of the fractions was as follows: lanes 1 and 14, ChCC49 Fab; lanes 2-13 correspond to fractions 3, 8, 10, 12, 14, 16, 18, 19, 20, 22, 23 and 24 respectively and lanes 15-24 correspond to fractions 25-33 and 35, respectively.

Both the resulting dimer and monomer pools were concentrated to 300 µL with centricon 30 devices and dialyzed overnight against 20 mM (1,3-bis[tris(hydroxymethyl)-

15   methylamino]propane (Bis-Tris propane)-Cl pH 6.8 buffer and applied to the Mono Q HR5/5 column for the monomer and dimer purification. The same gradient program was used as described earlier with buffer B being 20 mM Bis-Tris propane-Cl pH 6.8 in 0.5 M NaCl. The active Mono Q fractions were pooled and concentrated to 130 µL. The sample was applied to the Superdex 75 HR 10/30 column to verify purity of the monomer and exchange the buffer to

20   PBS.

<u>Antibody Competition</u>

Using purified monomer and dimer, it was possible to quantitate them accurately (Table 1) and compare them directly in a competition assay to a Fab, which is monovalent, and the CC49 whole antibody, which is divalent. The results of this study are given in Figure 13. The

25   results indicate the dimer and whole IgG have similar activity. These results further support the data of the existence of the dimer, indicating the dimer is folding in a way that gives two active, accessible binding sites.

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Table 1: Characterization of CC49 Antibody Species

Amino Acid Composition	SCFV UHM 8.1	Fv2 8.1	SCFV UHM 5.8	Fv2 5.8	SCFV UHM 5.2	Fv2 5.2	SCFV UHH	ChCC49 V_L[1]	ChCC49 V_H (83CH1)[1]	CC49 V_L[2]	CC49 V_H[2]
-Ala	20	40	20	40	20	40	21	12	15	10	24
-Cys	4	8	4	8	4	8	4	5	5	5	12
-Asp	18	36	20	40	21	42	19	9	8	11	18
-Glu	9	18	10	20	11	22	9	10	6	9	22
-Phe	8	16	8	16	8	16	8	7	8	7	19
-Gly	17	34	17	34	17	34	18	13	17	13	21
-His	2	4	2	4	2	4	2	2	4	2	11
-Ile	6	12	6	12	6	12	8	4	3	7	15
-Lys	24	48	24	48	24	48	22	16	14	15	32
-Leu	21	42	21	42	21	42	18	20	15	17	23
-Met	2	4	2	4	2	4	3	1	1	2	6
-Asn	7	14	7	14	7	14	8	7	9	10	21
-Pro	9	18	10	20	11	22	9	11	11	11	34
-Gln	15	30	15	30	15	30	14	14	10	11	21
-Arg	4	0	4	8	4	8	5	5	4	6	7
-Ser	35	70	35	70	34	68	32	35	32	35	47
-Thr	16	32	16	32	16	32	17	15	20	18	40
-Val	16	32	16	32	16	32	16	18	20	14	41
-Trp	5	10	5	10	5	10	5	3	4	4	9
-Tyr	15	30	16	32	16	32	15	13	9	13	13
TOTAL	253	506	258	516	260	520	253	220	215	220	436
E_{280}	47,980	95,960	49,320	98,640	49,320	98,640	47,980	68,880		215	750
E_{230} 0.1%	1.73	1.73	1.74	1.74	1.72	1.72	1.73	1.46		1.49	
MW[3]	27,749	55,498	28,369	56,738	28,623	57,246	27,736	24,125	22,956	24,294	48,037
pI	8.12	8.29-8.46	5.84	6.10-6.38	5.23	5.35	6.39	8.67	9.22	8.21	7.22

[1] The MW of the ChCC49 Fab from these components is 47,081 daltons.
[2] The MW of the CC49 IgG1 from these components is 144,662 daltons. Carbohydrate weight was not included.
[3] The values given are adjusted for disulfide bond formation between Cys residues.

The activity of the purified CC49 scFv5.2 was compared with the ChCC49 Fab in a competition assay (Figure 14) and the scFv was found to be equivalent to the Fab. This indicates one can add certain features to the carboxyl terminus of a scFv construction, as in this case the addition of Pro-Glu-Asp-Pro-Glu-Asp-Tyr-Asp, that do not affect the binding site. Therefore,

5 other modifying sequences can be contemplated which alter the overall characteristics of the molecule.

<u>Amino-Terminal Amino Acid Analysis</u>

The amino terminal sequencing data gave the following result: NH2-?-I-V-M-S-Q-S-P-S?-T?-L. The first residue couldn't be unequivocally assigned, but its positioning indicates

10 that signal peptidase had cleaved at the end of the *pel* B signal sequence, and released a correctly processed mature product. The residues indicated at positions 2-11 correspond to the sequence predicted by the DNA sequence (See Figure 8). The yields for Ser and Thr at positions 9 and 10 were low, but in general, Ser and Thr yields are known to be lower than for the other amino acids. Based on these data, one can conclude that the *pel* B signal sequence is effective

15 in directing the secretion of the single chain products to the *E. coli* periplasm. It is apparent that one need not be limited to using the *pel* B leader, and that the same results, as far as processing and secretion of the product is concerned, can be obtained by using other signal sequences such as *omp* A and *pen* PC, and others. One can include these sequences in the PCR oligo when making the construction upstream from the mature protein.

20 Temperature Stability Study of CC49 Fv2 8.1

Approximately 3 µg aliquots of CC49 Fv2 8.1 kept at 37°C were analyzed at intervals over 24 hours by gel filtration chromatography. A Superdex 75 HR10/30 column was utilized, using PBS as the mobile phase, at a flow rate of 0.5 mL/min.
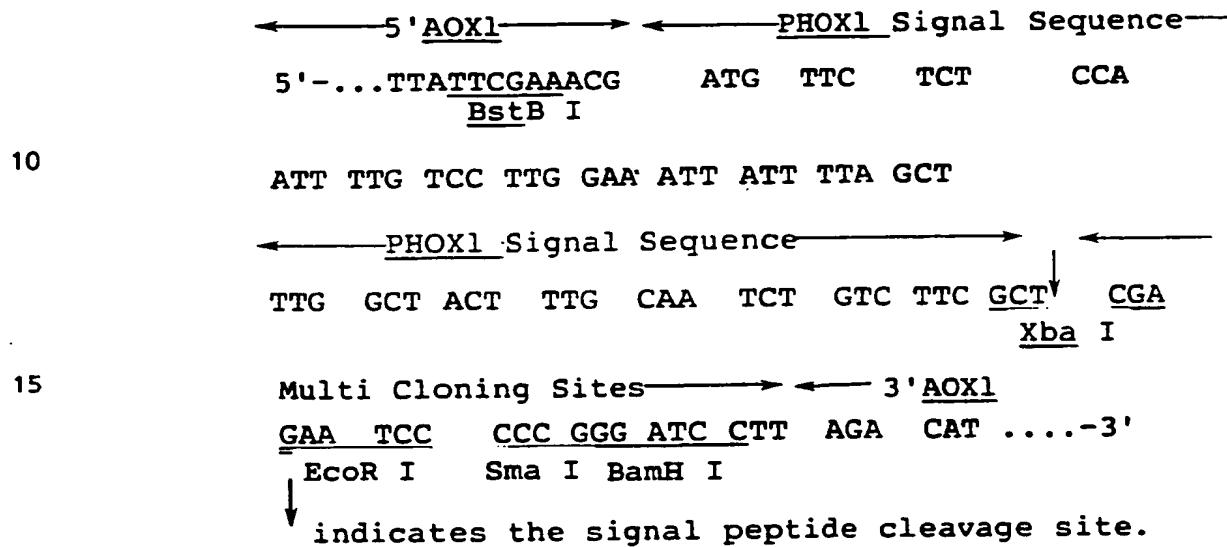
The results of samples taken at various time points over 24 hours at 37°C indicated

25 that the Fv2 remained intact (eluted at the same position as the starting material), and that no degradation products were observed.

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<u>Example 2</u> Cloning of CC49 Single Chain Antibody Species into the Yeast *Pichia pastoris*

For expression of single chain antibody species in yeast, the strain *Pichia pastoris* GTS115 *HIS4* (NRRL Y-15851) carrying a defective histidine dehydrogenase gene was used as a host cell. The plasmid pHIL-S1, available from Phillips Petroleum Company, was used as the expression plasmid modified as described below. The original sequence and features in the 5' region of pHIL-S1 are as follows:

```
←──────5'AOX1──────→  ←──────PHOX1 Signal Sequence──

5'-...TTATTCGAAACG      ATG  TTC  TCT      CCA
        BstB I


ATT TTG TCC TTG GAA ATT ATT TTA GCT


←──────PHOX1 Signal Sequence──────→   ←───────

TTG  GCT  ACT  TTG  CAA  TCT  GTC  TTC  GCT↓  CGA
                                            Xba I


Multi Cloning Sites──────→ ←──── 3'AOX1
GAA  TCC    CCC GGG ATC CTT  AGA    CAT ....-3'
 EcoR I      Sma I BamH I

↓
  indicates the signal peptide cleavage site.
```

<u>DNA Sequencing</u>

The dideoxy chain termination method of Sanger (Sambrook et al. *supra*) was used for DNA sequencing of the CC49 scFv genes present in pPY21 and pPY22. A Sequenase™ sequencing kit (United States Biochemical, Cleveland, OH) was used for the sequencing reactions, according to the manufacturer's directions. The radiolabel used was α-P32 dATP (3,000 Ci/mmol) obtained from Amersham (Arlington Heights, IL), lot no. AC9048. Double loadings were done using a BRL Model S2 sequencing apparatus and a premixed 6% Gel-Mix sequencing gel (BRL). After electrophoresis, gels were dried for 30 min using a Model 483 Slab Gel Dryer (Biorad, Richmond, CA) and exposed to Kodak X-OMat film (Sigma Chemical, St. Louis, MO) for 1-24 h. The sequencing oligonucleotides used for priming (0.5 pmol each) were:

1)   AOX1P (23-mer), 5'-TTTAACGACAACTTGAGAAGATC-3';

2)   TAGVLFR2 (20-mer), 5'-TGGTACCAGCAGAAACCAGG-3';

3)   TAGVLCDR3 (Mixed 22-mer),
      5'-GTCAGCA(AG)TATTATAG(CT)TATCC-3' and;

4)   TAGVHCDR2 (21-mer), 5'-ATGGATTGGATATTTTTCTCC-3'

<u>Construction of pPY1</u>

A clone of *E. coli* AG1 transformed with pHIL-S1 was used to isolate purified pHIL-S1 plasmid using Qiagen's midi plasmid prep procedure (Chatsworth, CA). Three

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micrograms of purified pHIL-S1 were treated with Sst I (Biological Research Laboratories, BRL) (10 units) in a volume of 40 µL for 45 minutes and then 2 µL of 1.5 M NaCl and 20 units (2 µL) of Sal I (BRL) were added (see Figure 15A). After the reaction proceeded for about 45 minutes, the sample was loaded onto a (16 X 18 cm) 4 percent polyacrylamide gel (PAG).

5   In a separate reaction, pBluescript II KS + (5 µg) purchased from Stratagene, was treated with Sst I (12.5 units) in a volume of 40 µL for 45 min. (see Figure 15B). The buffer was then modified to favor Sal I activity, by adding 2λ of 1.5 M NaCl, and then 25 units of Sal I were added. After another 45 min at 37°C, this sample was also loaded on the 4 percent PAG. DNA fragments were separated by electrophoresis at 90 volts over 2.5 h.

10   pHIL-S1 gave 2 fragments, a larger one at 5.9 Kbp containing the ampicillin resistance gene and a smaller fragment at 2.5 Kbp containing most of the 5'AOX1 region and multiple cloning site. Both fragments were cut out of the gel and isolated by electroelution and ethanol precipitation.

          For the pBluescript II digest, 2 bands were visualized, one corresponding to the vector piece at about 2.8 Kbp and a small fragment from the multiple cloning site. The 2.8 Kbp 15 fragment was recovered as above. To make pPY1 (Figure 15C), the pBluescript II Sst I/Sal I 2.8 Kbp fragment (~100 mg) was used in a ligation reaction with the 2.5 Kbp pHIL-S1 fragment (~100 mg). The ligation kit components were from Stratagene, while the T4 DNA ligase was a New England Biolabs product. Ligation commenced at 18°C, with a cooling gradient down to 20 4°C overnight. E. coli SURE (Stratagen, LaJolla, CA) was transformed with this ligation mix (1 µL and 5 µL samples). After overnight growth on Luria Broth (Sambrook et al., Supra) plates containing 100 µg/mL ampicillin, uncolored colonies (plasmids containing inserts) out--numbered blue colonies (without inserts) by roughly 10:1. Five of the uncolored colonies were picked for plasmid screening with Sst I and Sal I. All 5 gave the restriction pattern expected 25 based on the plasmid construction (2 bands at 2.8 Kbp and 2.5 Kbp). The plasmid from a clone designated #4 was tested further and found to have the correct sites for BstB I, Xho I (2 sites), EcoR V and BamH I, and was thus picked as the representative clone for pPY1.

Construction of pPY2

          Using the plasmid mini prep DNA of pPY1 from above, 2.5 µg were treated with 30 20 units of EcoR V (BRL) in a reaction volume of 45 µL at 37°C for 30 min. A single linearized DNA fragment at 5.3 Kbp was purified from a 3.75 percent PAG as described above. A linker DNA fragment with single EcoR I and Not I sites was then ligated into the blunt ended EcoR V site as follows.

          The oligos ECONOT, a 20-mer: 5'-GAATTCTTAGCGGCCGCTTG-3' for the top 35 strand and TONOCE, a 20-mer: 5'-CAAGCGGCCGCTAAGAATTC-3' for the bottom strand were synthesized and purified for annealing. A 60 fold molar excess of annealed linker over the EcoR V treated pPY1 fragment (5.7 X 10^{-14} moles -20 mg) were ligated using T4 DNA ligase and temperature conditions as above. Competent E. coli AG1 cells were transformed with aliquots

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(1 and 5 µL) of this ligation mix and plated (75 or 150 µL) onto LB agar plates containing 100 µg/mL ampicillin. Twelve clones were picked for plasmid screening with *Eco*R I. Out of these, 10 clones had the correct two *Eco*R I fragments of roughly 600 bp and 4.7 Kbp. Four of these clones were selected for DNA sequencing of their plasmids for verification and

5   orientation of the linker sequence. The oligo used for the sequencing (0.5 pmol) was ECOVNHESEQ, a 19-mer: 5'-TGCGCATAGAAATTGCATC-3'. Two of the plasmids had the correct sequence and desired orientation, one was chosen as the representative pPY2 clone (see Figure 15D).

<u>Construction of pPY21 and pPY22</u>

10   A common vector derived from pPY2 was used to generate both pPY21 and pPY22. Two micrograms of pPY2 mini-prep DNA were treated with 24 units of *Bst*B I (New England Biolabs) in a 20 µL reaction volume at 65°C for 15 min. At this point, 1.5 M NaCl was added (1.2 µL) with 12 units (1.2 µL) of *Bam*H I (New England Biolabs) and the reaction allowed to proceed for 15 min at 37°C. This sample was loaded on a 4 percent PAG, and the pPY2

15   fragment with *Bst*B I and *Bam*H I ends of roughly 5.2 Kbp was recovered by electroelution and ethanol precipitation.

The PCRs to generate 2 CC49 scFv inserts for the pPY2 vector were performed as follows. Oligos (100 pmol each) targeting pSCFV UHM5.2, prepared as described in Example 1 (5 ng) were:

20   1)   PY49VLI, a 92-mer,
                    5'-ACATTTCGAAACGATGCTTTTGCAAGCTTTCCTTTTCCTTTTGGCTG
                    GTTTTGCAGCTAAGATATCTGCTGACATTGTGATGTCACAGTCTC-3'; and
            2)   pPY523, a 36-mer,
                    5'-AAATGGATCCTATTAGTCATAGTCTTCAGGGTCTTC-3'.

25   *Taq* polymerase was purchased from Perkin Elmer Cetus (Norwalk, CT) and used in conjunction with the GeneAmp™ kit, according to the manufacturer's directions. The conditions for the PCR were: 30 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 1 min. On the final cycle, the last elongation step (72°C) was 2 min. An MJ Research (Watertown, MA) thermal cycler was used.

30   For the PCR of the second CC49 scFv insert, oligos (100 pmol each) targeting pSCFV UHM8.1 (see example 1) (5 ng) were:
            1)   PY49VL1, as above; and
            2)   PSC49VH, a 40-mer,
                    5'-ATGTGGATCCCTATTAGTAGGAGACGGTGACTGAGGTTCC-3'.

35   The reaction was set up and carried out as described for pSCFV UHM5.2, above. The PCR products were purified free of reactants by electrophoresis in a 4 percent PAG. The desired bands from the pSCFV UHM5.2 and pSCFV UHM8.1 targets were visualized at the expected

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875 bp and 854 bp sizes respectively. The DNA was recovered by electro lution and ethanol precipitation.

The PCR inserts were prepared for ligation by trimming the ends off of each with *Bst*B I at the 5′ ends and *Bam*H I at the 3′ ends, using the same reaction conditions as described
5   above for the pPY2 vector. After this treatment, the DNA inserts were again purified using a 4 percent PAG, subsequent electroelution and ethanol precipitation. The DNA inserts generated from pSCFV UHM5.2 and pSCFV UHM8.1 were used to make pPY21 and pPY22, respectively (Figure 15E). The ligation reactions regenerating pPY21 and pPY22 were set up (20 µL total volume) using 100 mg of pPY2 vector for each, and a 1:1 stoichiometric equivalent
10   of the prepared PCR inserts from pSCFV UHM5.2 and pSCFV UHM8.1 respectively. The ligation kit components and T4 DNA ligase were from Stratagene and used according to their directions. The ligation reactions were incubated at 15°C for 2 hours, and 2 µL from each used to transform *E. coli* AG1 competent cells. Samples were plated onto LB agar containing 100 µg/mL ampicillin. Seven clones each for pPY21 and pPY22 were picked for screening of their
15   plasmid DNAs, using the *Bst*B I-*Bam*H I double digest described above. A single clone (#6) was obtained with the correct DNA fragments for pPY21, while four clones (#11-14) were obtained for pPY22. The clone for pPY21 and pPY22 clone #12 were selected for DNA sequencing of the insert DNAs containing the CC49 scFv constructions. Both clones contained the correct sequence for CC49 scFv, 5.2 (pPY21) and 8.1 (pPY22), respectively, and were used for the final
20   expression plasmid constructions.

The DNA and amino acid sequences of CC49 scFv in pPY21 and pPY22 are given in Figure 16. In these scFv constructions, the V_L gene came first followed by the V_H and was joined by a 25 amino acid linker which is in bold and underlined. The oligonucleotides used as DNA sequencing primers are shown above the DNA sequence lines. Restriction enzyme sites are as
25   indicated, and underlined. The SUC2 signal peptide is underlined; the putative first amino acid of these mature products is indicated by (+ 1). Complementarity determining CDRs regions segments for the CC49V_L and CC49V_H are italicized in bold. Sequences for pPY21, giving negatively charged proteins (CC49, scFv5.2, Fv(2n)-5.2) are indicated by sequence A; and sequences for pPY22, giving close to neutrally charged scFv products (CC49, scFv8.1P,
30   Fv-(2n)-8.1P) are represented by sequence B.

Construction of pPY31 and pPY32

The *Sst* I-*Sal* I DNA fragments of pPY21 and pPY22 containing the CC49 scFv constructions were isolated and purified, to be used as inserts with the pHIL-S1 vector fragment (5.9 Kbp) already described above. Three micrograms of pPY21 and pPY22 plasmid mini-prep
35   DNAs from above (same clones as were sequenced) were treated with 9 units each of *Sst* I(BRL) in a 30 µL reaction at 37°C for 20 min. The samples were electrophoresed on a 4 percent PAG. The resulting bands at 3.2 Kbp (containing the DNA of interest) and at 2.8 Kbp were isolated together, since the resolution was not adequate for good resolution on the PAG. These pairs of

DNA inserts were then ligated with the pHIL-S1 *Sst* I-*Sal* I vector fragment (100 mg) in a 1:1

stoichiometric ratio, in a 20 µL reaction volume for 2h at 15°C to form pPY31 and pPY32

(Figure 15F). Competent *E. coli* AG1 cells were transformed with 2.5 µL aliquots of the ligation

mixes and plated onto LB agar plates containing 100 µg/mL ampicillin. Six potential clones each

5   of pPY31 and pPY32 were selected for plasmid screening. The resulting mini-prep DNAs were

digested with an *Sst*I-*Sal*I double digest, as described above for the insert preparation. Three

clones had the correct 2 bands (3.2 Kbp and 5.9 Kbp) for pPY31, one was chosen as the pPY31

representative. Two clones had the correct bands for pPY32 and one of them was chosen as the

representative for transformation into *Pichia*.

10   <u>Transformation of *Pichia pastoris* GTS115 with pPY31 and pPY32</u>

The procedure of Becker and Guarente for [*Methods in Enzymology*, <u>194,</u> 182

(1990)]. electroporation of yeast cells was followed with some modifications, as described

below, to introduce the CC49 scFv genes into *Pichia*. Yeast extract peptone glucose (YEiD)

medium (125 mL) was inoculated for overnight culture at 30°C from a stock of *P. pastoris*

15   GTS115 stored at 4°C. The cells were harvested the next day when the O.D.$_{600nm}$ was between

0.8-0.9. After washing with sterile water and 1M sorbitol, the cells were resuspended in ice-

-cold 1 M sorbitol (150 µL) and held on ice. Aliquots of pPY31 or pPY32 (2 µg/µL) linearized

with *Bgl* II, totaling 5 µg and 10 µg were added to 40 µL each of the *Pichia* electro competent

cells and transferred to pre-chilled (on ice) 0.2 cm sterile electroporation cuvettes (Biorad).

20   Each sample was pulsed at 1.5 kV, 25 µF and 200 ohms, using a Biorad Gene Pulser with Pulse

Controller. The time interval of the pulsers was 4.7-4.8 msec. Immediately after each pulse,

1.0 mL of cold 1M sorbitol was added to the cuvette, and three RDB plates for each of the 4

transformations (see U.S. Patents 4,855,231, 4,879,231 and 4,808,537) were plated (200 µL

each). Plates were incubated at 30°C. Four days later, 36 colonies (numbered 1-36) transformed

25   with pPY31 and 35 colonies (numbered 37-71) transformed with pPY32 were randomly picked

and inoculated into 2.0 mL of BMGY media and streaked on MD and MM plates. All were

grown at 30°C. BMGY is buffered minimal glycerol-complex medium containing 1 m potassium

phosphate (pH 6.0); 13.4 g/L yeast nitrogen base with ammonium sulfate; 400 µg/L biotin;

10 g/L yeast extract; and 20 g/L peptone. MD is a medium containing 13.4 g/L yeast nitrogen

30   base; 400 µg/L biotin; and 20 g/L dextrose. MM is the same as MD with 5 mL/L of methanol

substituted for the dextrose.

Clones able to grow on MM pates are not *AOX*·, implying that the Bgl II fragment

did not incorporate at the *AOX*1 locus, but at some other chromosomal location. The MM

plates have only methanol as a carbon source. After 2 days of growth in the BMGY medium, 9

35   potential *AOX*· mutant lines (observed by slow growth on the MM plate, 3 for PPY31,

designated Nos. 4, 31, 32 and 6 for pPY32, designated Nos. 39, 44, 60, 61, 65 and 71 and an

AOX· control (Clone No. 11, from the pPY31 set) were transferred to BMMY medium (2.0 mL

each). BMMY medium is the same as BMGY with 5 ml/L methanol substituted for glycerol.

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Aliquots of methanol (20 μL) were added to each tube, roughly every 10-14 h. Samples (100 μL) from each culture were taken 32 h after methanol induction and the biological activity determined by a competition assay described in Example 1. SDS-PAGE 10-20 percent gradient gels were run on 10 μL aliquots taken 32 and 100 h post induction. Western transfer and

5   detection by biotinylated FAID14 was also done for the 100 hour samples.

The relative anti TAG-72 activities were evaluated for the methanol slow (AOX-) clones, along with some normal growing clones on MM medium. Biotinylated CC49 was used as the competitor in a competition assay. The results are presented in Figure 17, where the percent competition for a given sample was calculated based on $OD_{405nm}-OD_{450nm}$ readings of

10   the average of triplicate samples assayed using the following formula:

$$\frac{zero\ competition\ -\ Sample\ \ \ \ x10^0}{zero\ competition\ -\ 100\%\ competition} = \%\ competition$$

The zero competition value was obtained from a 0.2 micron filtered BMMY medium sample,

15   while the 100 percent competition value was obtained using a 25 μL sample of CC49 IgG at 5 μg/mL. The *Pichia* samples for assay were prepared by microcentrifugation of the culture sample and filtration through a 0.22 μ filter. For pPY31 the results indicated that the methanol slow clones 4 and 32 competed very well (Fig. 17) as did clone 31, which by observation of growth on the MM plate could not be categorized as methanol slow. Clones 11 and 17 were

20   producing little or no scFv. For pPY32, the methanol slow clones 39, 60, 61, 65 and 71 all gave excellent competition. Clones 38, 44 and 50 gave little or no competition.

The SDS-PAGE-Western data corroborated the competition ELISA results. The main product of the crude *Pichia* culture supernatant observed on SDS-PAGE-Western was the scFv product. The *Pichia* products correlated well with the previously obtained *E. coli* product

25   equivalents in terms of size on the SDS-PAGE and activity in the competition assay. In the case of the pI 8.1 *Pichia* products, a very slightly slower mobility was observed relative to the Western positive band representing the *E. coli* CC49 scFv I8.1 product. This is due to the increase in molecular weight of the *Pichia* product due to the carboxyl terminal tyrosine residue weighing approximately 78 daltons more than the corresponding serine residue at the same

30   position in the *E. coli* product.

Purification of Single Chain Antibody Species from *P. pastoris* GTS115 pPY31 Culture

Pichia pastoris carrying the plasmid pPY31 produces a CC49 single chain antibody having a pI of about 5.2. Starter cultures (20 mL BMGY medium) was inoculated (Clone #4 above) and grown for 26.5 hours at 30°C. This was used then to inoculate 800 mL BMGY

35   medium in a 2.8 L Fernbach flask. The culture was grown at 30°C for 24 hours with shaking at 150 rpm. The *Pichia* cells wer pell ted at 10°C using 2 tubes and a G53 rotor (Sorvall) run at 5,000 rpm for 10 minutes. The cells from each tube were aseptically resuspended in BMMY

medium (40 mL) to which 75 μL of methanol were added and continued to shake at 30°C.
Methanol (200 μL) was added approximately every 12 hours over a 36 hour gr wth period.

Culture supernatants (containing the single chain antibody products) were
harvested by spinning the cultures at 5,500 rpm in a SS34 rotor (Sorvall) for 5 minutes. The

5   supernatant (about 66 mL) was further clarified by filtering through a 0.22 μ pore size filter
device (Nalge) and then concentrated to 10 mL using a Centriprep 10 device (Amiccn). Half of
this material was applied to a Superdex 75 HR26/60 gel filtration column (Pharmacia), at a flow
rate of 1.3 mL/min using PBS as the buffer. The gel filtration profile (See Figure 18) gave three
pools of single chain species indicating the formation of monomeric single chain antibody

10 (scFv, fraction 19-23), dimers (scFv2, fractions 13-15) and multimeric forms (scFv(2n) where n > 1,
fractions 8-10). On the SDS-PAGE insert, lane "S" indicates the identical purified protein
standard CC49 scFv5.2 derived from *E. coli* pSCFV UHM5.2 (Example 1). In the processing of
running SDS-PAGE, the non-covalent interactions in a dimer or multimer are caused to break
apart, showing that the dimer and multimer forms correspond to the same size as the basic

15 monomeric unit.

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TABLE I

GEL FILTRATION ANALYSIS OF CC49 SCFV PI = 5.2 SPECIES[1]

Single Chain Antibody Species	Elution Time (min.)	Elution Volume (mL)	Kav[2]	Predicted MW (Kd)	Observed MW (Kd)	Notes
A. Monomer: scFv	21.1	12.66	.320	28.6	30.5	Derived from fractions 26 and 27, Figure 2A, pool 1
B. Dimer: Fv2	18.8	11.28	.237	57.2	68	Derived from fractions 41 and 42, Figure 2B, pool 2
C. Tetramer: Fv4	17.5	10.5	.191	114.5	120	Derived from fractions 40 and 41, Figure 2Ca, pool 3
D. Hexamer: Fv6	17.0	10.2	.173	171.7	180	Derived from fractions 42 and 43, Figure 2Cb, pool 4
E. Octomer: Fv8 and above?	-[3]	-[3]	-[3]	229.0	-[3]	Derived from fraction 44, Figure 2Cc

[1] Buffer used was PBS, at a flow rate of 0.6 mL/min. In each case, all peaks were indicative of a pure species with <10 percent of any contamination from other forms or products.

[2] Based on a void volume of 7.32 mL (thyroglobulin, MW=670 Kd) and a column volume of 24 mL. Kav values for the MW standards borine IgG at 158 Kd daltons, ovalbumin at 44 Kd daltons, myoglobulin at 17 Kd daltons and cyanocobalamin at 1,350 daltons were 0.178, 0.284, 0.381 and 0.644, respectively.

[3] All of the above samples were run twice, except this one which was only run once. Using a different Superdex 75 column at that time the elution times for the species scFv, Fv2, Fv4, Fv6 and this putative Fv8 were 16.74, 14.10, 12.78, 12.46 and 12.2 minutes, respectively. No gel filtration standards were run at the same time so that a MW for the putative Fv8 could not be determined.

Each of th single chain antibody pools was further purified by us of a Mono Q HR5/5 anion exchange column as described in Example 1. Each of the various species was then re-chromatographed on an analytical gel filtration column (Superdex 75 HR10/30, Pharmacia-LKB). Table II gives the elution time, elution volume, Kav values and molecular
5   weights determined for the monomeric and various multimeric forms. The predicted molecular weights were predicted by DNASTAR™ protein titrate computer program using the amino acid composition of the polypeptide.

The Anion Exchange Chromatography and SDS-PAGE are given in Figure 19. Relevant parts A, B, and C correspond to the monomer, dimer and higher multimer forms of
10   the scFv species, respectively. The buffer system used and the gradient of Buffer B are also shown. The SDS-PAGE insert (D) has the following samples: molecular weight markers (lane 1); monomer and dimer (lanes 2 and 3); and E. coli derived CC49 scFv 5.2 standard (lane 4); tetramer, hexamer and putative octamer (lanes 5, 6, and 7, respectively) and an approximately 34 Kd impurity (lane 8) derived from fraction 45.

15   The IEF gel and Western, shown in Figure 20 indicate that the monomer and various multimer forms have distinct pI values. Both the E. coli and Pichia derived monomer forms, have a pI of 4.92 (derived from a graph of distance from cathode vs. pI of the standards), with a minor band at pI 4.97, which is the same as the pI for all multimers. That 2 bands seen in the monomer (lanes 3 and 9) support the hypothesis that there are two charge forms for the
20   monomer in equilibrium, where the pI 4.97 form has a tendency to dimerize. In Figure 20, lanes 1-7 represent bands stained with Coomassie Brilliant Blue R-250 and lanes 8-13 represent a Western transfer of samples corresponding to lanes 2-7. The pI markers (lane 1) were from Sigma: soybean trypsin inhibitor, pI 4.55; bovine β-lactoglobulin A, pI 5.13; and human erythrocyte carbonic anhydrase B, pI 6.57. Lanes 2 and 8 show purified CC49 scFv5.2 derived
25   from E. coli pSCFV UHM5.2; lanes 3 and 9 show the same product derived from Pichia. Lanes 4 and 10 show a sample of crude culture supernatant from Pichia containing all the CC49 single chain antibody species present. Lanes 5 and 11, 6 and 12, and 7 and 13 indicate the Pichia dimer, tetramer and hexamer species, respectively.

Purification of Single Chain Antibody Species of Pichia pastoris GTS115/pPY32

30   P. pastoris having the plasmid pPY32 produces CC49 single chain antibodies having a pI of about 8.1. The growth, harvesting and induction of the AOX1 promoter were the same as for the pPY31 clone above.

The purity of the 8.1 monomer and dimer (multimeric forms were not analyzed) was verified by SDS-PAGE. The IEF pattern of these molecules indicated that except for
35   carboxyl terminal Ser residue present in the E. coli derived CC49 scFv8.1 and Fv28.1, the corresponding Pichia products have the same pI values. In Pichia the carboxyl terminal residue is Tyr.

The N-terminal amin   acid sequencing results indicated that the invertase signal
sequence used in constructing these single chain antibodies was accurately being removed by
signal peptidase. For the CC49 scFv8.1P and Fv2-8.1P proteins, the following NH$_2$ terminal
residues were determined: Asp-Ile-Val-Met-Ser-Gln-Ser-Pro-Ser-Ser, which matches the
5   predicted amino acid sequence of the mature form, from the DNA sequence (see Figure 16).
For the CC49 Fv2-5.2 product, the same results were obtained as above, except that the first and
tenth amino acids could not be unequivocally assigned. Based on the above results with the
8.1 pl system, it is reasonable to assume for the 5.2 pl products that the unassigned residues are
the same.

10   From the competition ELISA results (Figure 21A and 21B), it can be observed that
the avidity of the single chain species, increases with higher multimer forms. It is possible that
the effects seen for the tetramer and hexamer are underestimated and that different
concentrations or densities of TAG-72 on the ELISA plate may influence the results. The
availability of antigen to some of the binding faces of the hexamer and octamer, for example,
15   may not be sterically available.

Example 3   Biodistribution Characteristics of ^{125}I Labeled CC49 Single Chain Fv Species

The biodistribution of four CC49 single chain antibody species, two monomeric
species: CC49 scFv with a pl of 8.1 obtained from *Pichia* (scFv 8.1P) and CC49 with a pl of 5.2 also
obtained from *Pichia* (scFv 5.2); and two dimeric species: CC49 Fv2 with a pl of 8.1 obtained
20   from *Pichia* (Fv2-8.1P) and CC49 Fv2 with a pl of 5.2 obtained from *E. coli* (Fv2-5.2), was
determined. This was compared with ChCC49 Fab and IgG. The single chain species were
labeled with ^{125}I using the Iodo-Beads™ (Pierce, Rockford, IL) method using N-chloro-
benzenesulfonamide derivatived polystyrene beads (Iodo-Beads™, Pierce, Rockford, IL). To
label the antibodies, initially 3 Iodo-Beads™ were incubated at room temperature for 5 minutes
25   in PBS and 150 µCi carrier-free I-125 (Nordion, 100.9 µCi/µL). At this point, the antibody (0.3 mg
in PBS) was added and allowed to react for 8 minutes. Unincorporated isotope and reaction
by-products were removed by gel filtration chromatography (Pharmacia Superdex 75 HR10/30
column), using PBS as the buffer at a flow rate of 0.6 mL/minute. An aliquot of the purified
protein peak was measured in each case at A$_{280nm}$ and quantitated according to the E$^{1\%}$$_{280nm}$
30   values as described in Example 1 for the respective proteins. Levels of radioactivity were
determined by measurement in a Capintec Counter, set for reading I-125.

SDS-PAGE and IEF analyses were performed as previously described. Gels were
dried and radioactive bands detected by exposure to Kodak XAR X-OMAT film. The observed pl
values for the 8.1P species, as determined by IEF are 7.1 and 7.25 for monomer and dimer,
35   respectively, and for the 5.2 species, 4.9 and 5.0 for the monomer and dimer, respectively.
Differences between the calculated values (after which the species are officially called) and the
observed values indicate that the tertiary structure of these molecules play a role in charge-
charge interactions and/or hydrogen bonding which will determine the observed pl values.

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Five test groups, each containing 5 female nu/nu (CD-1) mice (obtain d from
Charles River Breeding Laboratories) bearing LS174T human tumor xenografts, were used to
measure the biodistribution of ^{125}I labeled antibody species. The biodistribution of the
compounds was measured at various time intervals over 48 hours, after intravenous (tail vein)

5   injection of the compounds. Animals were housed and identified by group as outlined in
Section 4.0 of Standard Operation Manual for the Care of Laboratory Animals (SOP-PTG). At
the appropriate time, the mice were euthanatized and the radioactivity remaining in samples
of blood, liver, spleen, kidney, tumor, lung, GI tract, tail, and remaining carcass were quantifi d
as outlined in Section 4.0 and Section 5.0 of SOP-PTG. The LS174T tumors were produced in the

10   mice according to the procedure set forth in Section 2.0 of SOP-PTG.

For metabolite studies, groups of Balb/c mice were injected intravenously with
1 µCi of one of the antibody species. Mice from each group were anesthetized after
30 minutes, 2h and 5h, urine removed from the bladder, urine counted for radioactivity and
then kept frozen at -70°C until HPLC analysis. A Pharmacia Superdex 75 gel filtration HR10/30

15   column was used for chromatography, using PBS as the eluent at 1.5 mL/minute. The volumes
of the injected urine varied from 20-80 µL. The column eluent passed through a dual detector
for absorbence at 280nm and for radioactivity.

Gamma Counter #2 (Searle/ND-66) was used to measure radioactivity in standards
and tissue samples. Biodistribution of radioactivity in the collected tissues was analyzed using

20   template TEMPL as outlined in Section 5.0 of SOP-PTG. Experimental outliers were treated as
outlined in Section 6.0 of SOP-PTG.

Table IV gives the amount of protein and activity of each species injected into the
mice for biodistribution studies.

Tables IV, V, VI, VII, VIII, and IX give the biodistribution of ^{125}I CC49 species of

25   scFv5.2, Fv2-5.2, and scFv8.1P, Fv2-8.1P, ChFab and IgG, respectively, based on percent injected
dose per organ. Percent injected dose per organ is calculated by determining the ^{125}I in counts
per minute (CPM) in each tissue, divided by the injected dose (CPM) multiplied by 100 to give
percent.

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TABLE III

Summary of Protein and Radioactivity Amounts Used to Inject Nude Mice Bearing LS174T Tumor Xenografts

Study No.	scFV5.2 N9103	FV2-5.2 N9103	scFV8.1P N9105	FV2-8.1P N9105	Ch Fab N9102	IgG. N9101
reg. protein Per mouse	6.85	7.75	5.15	3.85	27.25	21.95
uCi I-125 per mouse	1.12	1.60	1.00	0.75	12.5	4.32
Percent Activity (as determined by BSM binding)	64	895	91	68	64	96
Specific Activity (μCi/μg)	0.163	0.21	0.19	0.19	0.46	0.20

TABLE IV

Biodistribution of ^{125}I Injected as ^{125}I-CC49 scFv Monomer, pI 5.2 (scFV5.2)
Values as Percent Injected Dose/Gram (n=5)

Tissue	20 Minutes		2 hours		5 Hours		24 Hours		48 Hours	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	5.87	0.58	2.43	0.39	1.53	0.22	0.02	0.01	0.01	0.00 n=4,a
Liver	4.08	0.42	1.77	0.16	1.16	0.08	0.28	0.05	0.17	0.02
Spleen	3.35	0.80	1.77	0.09	1.17	0.13	0.19	0.03	0.14	0.03
Kidney	155.25	17.46	51.58	6.37	34.25	0.53	15.76	1.33	9.62	1.69
Tumor	2.80	0.34	2.24	0.13 n=4,a	1.72	0.25	0.58	0.09	0.40	0.08
Lung	5.72	0.88	2.91	0.72	1.04	0.27	0.13	0.11	0.01	0.00
Tumor Weight	0.20	0.04	0.19	0.05	0.27	0.07	0.13	0.07	0.17	0.04

TABLE V
Biodistribution of ^{125}I Injected as ^{125}I- CC49 scFv Dimer, pI 5.2 (Fv2-5.2)
Values as Percent Injected Dose/Gram (n=5)

Tissue	20 Minutes		2 hours		5 Hours		24 Hours		48 Hours	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	29.47	3.26	12.47	1.81	3.95	1.37	0.04	0.02	0.01	0.01
Liver	10.24	0.35 n=4,a	5.51	0.79	2.87	0.54	0.55	0.04	0.30	0.09
Spleen	9.63	2.56	5.45	0.73	2.31	0.30	0.34	0.08	0.22	0.12
Kidney	30.56	0.89 n=4,a	15.85	1.07	8.63	1.22	3.12	0.26	2.07	0.51
Tumor	3.57	0.16 n=4,a	8.91	1.58	8.52	2.35	7.66	1.74	4.96	2.08
Lung	8.65	0.78	4.84	0.80	2.09	0.52	0.07	0.02	0.01	0.01
Tumor Weight	0.17	0.02	0.25	0.11	0.27	0.04	0.18	0.10	0.14	0.06

TABLE VI

Biodistribution of ^{125}I Injected as ^{125}I-CC49 scFv Monomer, pI 8.1 (scFv8.1P)
Values as Percent Injected Dose/Gram

Tissue	20 Minutes n=3		2 hours n=5		5 Hours n=5		24 Hours n=5		48 Hours n=5	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	5.31	0.77	2.43	0.81	2.36	0.75	0.01	0.00	0.01	0.00
Liver	5.80	0.96	1.30	0.33	1.05	0.41	0.07	0.01	0.04	0.01
Spleen	3.79	0.03 n=2,a	1.81	0.51	1.42	0.12 n=4,a	0.05	0.02	0.02	0.01
Kidney	150.74	34.76	5.91	0.71	3.05	0.70	0.37	0.03	0.23	0.02
Tumor	3.06	0.38	2.19	0.28	2.09	0.35	0.64	0.06	0.40	0.03 n=4,a
Lung	6.75	1.01	7.25	0.86 n=4,a	1.34	0.32	0.02	0.01 n=4,a	0.00	0.00
Tumor Weight	0.24	0.03	0.16	0.04	0.25	0.10	0.18	0.06	0.16	0.03

TABLE VII
Biodistribution of ^{125}I Injected as ^{125}I-CC49 scFv Dimer, pI 8.1 (Fv2-8.1P)
Values as Percent Injected Dose/Gram

Tissue	20 Minutes n=4		2 hours n=5		5 Hours n=5		24 Hours n=5		48 Hours n=5	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	20.55	3.26	3.30	0.60	1.91	0.51	0.01	0.00	0.00	0.00
Liver	15.74	2.00	3.92	0.46	1.81	0.28	0.23	0.03	0.11	0.02
Spleen	10.52	1.94	3.71	0.82	1.73	0.17	0.19	0.07	0.09	0.04
Kidney	31.40	2.16	4.90	0.63	1.71	0.30	0.12	0.02	0.05	0.01
Tumor	1.70	0.45	3.18	0.89	2.23	0.40 n=4,a	1.56	0.35	0.93	0.18
Lung	5.77	1.26	3.05	0.86 n=4,b	1.15	0.33	0.03	0.04	0.00	0.00
Tumor Weight	0.25	0.13	0.21	0.08	0.35	0.08	0.14	0.02	0.15	0.05

TABLE VIII
Biodistribution of ^{125}I Injected as 125I ChCC49 Fab (Ch Fab), Values as Percent Injected Dose/Gram (n = 5)

Tissue	15 Minutes n=4		30 Minutes n=3		2 Hours n=5		5 Hours n=5		24 Hours n=5	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	17.11	3.85	6.06	0.63	4.68	0.42	1.96	0.39	0.16	0.02
Liver	6.82	0.45	4.32	0.38	2.78	0.58	1.19	0.26	0.43	0.02 n=4,a
Spleen	5.92	1.54	3.28	0.80	2.84	0.77	1.13	0.31	0.24	0.00 n=4,a
Kidney	154.17	22.39	167.64	9.52	23.81	7.39	5.46	1.23	1.29	0.06 n=4,a
Tumor	2.54	0.56	2.36	0.19	3.03	0.13	1.75	0.20	1.03	0.10
Lung	6.08	0.47	4.94	1.02	2.56	0.17	1.27	0.31	0.13	0.02
Tumor Weight	0.26	0.14	0.36	0.10	0.33	0.12	0.20	0.09	0.11	0.02

a One tissue value rejected due to Outlier Statistic >p-95 value.

TABLE IX
Biodistribution of 125I Injected as 125I-IgG
Values as Percent Injected Dose/Gram

Tissue	5 Hours n=5		24 Hours n=5		48 Hours n=4		120 Hours n=5	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	22.13	1.05	13.73	1.25	10.24	0.91	8.14	0.35 n=4,a
Liver	7.87	0.58	3.96	0.22 n=4,a	2.77	0.12	2.13	0.09 n=4,a
Spleen	5.06	0.45	3.05	0.62	2.14	0.22	1.56	0.26
Kidney	3.52	0.65	1.58	0.41	1.77	0.02 n=3.,a	1.53	0.12 n=4,a
Tumor	10.85	2.51	30.12	10.09	55.49	1.50 n=3.,a	58.21	12.56
Lung	6.04	0.56	3.80	0.62	3.11	0.39	2.18	0.34 n=4,a
Tumor Weight	0.19	0.03	0.17	0.03	0.17	0.02	0.18	0.07

a One tissue value rejected due to Outlier Statistic >p-95 value.
b One tumor value rejected-outside acceptable weight range.

The differences in pharmacokinetics for the various sizes and charges of the CC49 antibody species are demonstrated by the data in tables IV - IX. In general, the smaller the molecule, the faster it clears th blood. The 25-50 K dalton size molecules (all except the IgG) clear the blood within 24 hours. The negatively charged Fv2-5.2 dimer has the greatest amount in the blood at 2 hour (Table V). Both dimers accumulate in the tumor to the largest extent over a 24 hour time period (Tables V and VII), excluding the IgG.

A major difference between the antibody species is observed in retention by the kidneys. The negatively charged monomer species, CC49 scFv5.2, clears the most slowly (Table IV). This is surprising in that the negative charge should have prevented electrostatic interaction with the negatively charged glomerular cells of the kidney. The nominally neutral charged species, scFv8.1P cleared more quickly (Table VI). This trend was also true for the two dimers, but clearance was much better for these relative to the monomers. It is unexpected that the negatively charged dimer would have the best tumor localization values (Table V). The data also shows that a relatively small molecule (58 Kd) has staying power on the tumor. Both dimers out performed the monomers and chimeric CC49 Fab molecule in this regard.

Results from metabolic studies indicated that after injection with ^{125}I labeled scFv 5.2 and Fv2-5.2, only one radioactive metabolite was observed in the urine, free ^{125}I, as determined by gel filtration chromatography. No larger peptides are observed at any of the time points. These data indicate that dehalogenation is occurring within the body and that free I-125 is able to be readily excreted into the urine.

The tumor to tissue ratios 8 to 24 hours post injection indicated the dimers would be effective in radioimmunoguided surgery procedures. Surgery may be performed in a more timely fashion, rather than 2 to 3 weeks post injection, as is presently necessitated by the biodistribution kinetics of whole antibody.

Example 4   Construction, Purification and Characterization of Anti TAG-72 Single Chain Antibody Species with the Human Subgroup IV V_L

To provide for a rapid and convenient procedure to determine the effectiveness of the single chain dimers and multimers of the present invention, a unique octapeptide sequence -NH2-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH, called the FLAG™ peptide (a trademark of International Biotechnologies Inc., CT) was used. This sequence is recognized by International Biotechnology Incorporated's (IBI) mouse M2 monoclonal antibody. Placing the FLAG™ sequence on two annealing oligonucleotides in pSCFV UHH leads to a single chain antibody having the human subgroup 4 V_L and CC49 V_H with the FLAG peptide attached at the carboxyl terminus.

The final plasmid derived from pSCFV UHH containing FLAG™ is designated pSC49FLAG. The nomenclature for the single chain proteins produced from pSC49FLAG is as follows: for the monomer-H4L49HF scFv, for the dimer - H4L49HF Fv2 and for the tetramer-

-50-

H4L49HF Fv4. The "H4L" portion refers to the human Subgroup IV light chain V_L regi n, "49H" refers to the CC49 V_H and "F" indicates that the FLAG™, peptide is attached.

<u>Construction of pSC49FLAG</u>

A general outline for obtaining the plasmid pSC49FLAG is shown in Figure 22 and

5   the procedure described below.

a) <u>Isolation of pSCFVUHH Xho I/Nhe I Vector Fragment</u>

Approximately 5 µg of plasmid pSCFV UHH (Example 1) in 15 µL of water was used from the Magic Mini-prep system (Promega). To this was added 5.4 µL of 10X Buffer #2 (New England Biolabs), 45 units of Xho I (New England Biolabs), 15 units of Nhe I and 24 µL of water.

10   The reaction proceeded for 1 hour at 37°C. The sample was then loaded with running dyes on a 4 percent polyacrylamide gel, electrophoresed and purified by electroelution (Sambrook et al, supra). The obtained DNA pellet was dissolved in 20 µL of water.

b) <u>Construction of pATDFLAG</u>

Plasmid pSCFV UHH treated with Xho I and Nhe I from above was used in a

15   ligation reaction with annealed FLAG and FLAGNC oligos.

FLAGC:

5'-TCGAGACAATGTCGCTAGCGACTACAAGGACGATGATGACAAATAAAAAC-3'

FLAGNC:

5'-CTAGGTTTTTATTTGTCATCATCGTCCTTGTAGTCGCTAGCGACATTGTC-3'

20   Equimolar amounts (3 µM of each of the oligos) FLAGC and FLAGNC in a total volume of 62.5 µL) were mixed together using a ligation buffer diluted to 1X (Stratagene). The sample was heated to 95°C for 2 min and was allowed to cool to below 35°C over 10 min before use in the ligation reaction below.

	COMPONENT	AMOUNT
25	pSCFVUHH $XhoI/NheI$ vector (~100 ng)	1.0 µL
	ANNEALED FLAGC/FLAGNC	0.85 µL
	10X Ligation buffer	2 µL
30	T4 DNA Ligase	1 µL
	10 MM ATP	2 µL
	ddH₂O	13.2 µL

The reaction was carried out using the components and amounts indicated above.

35   Starting at 18°C, and then being cooled gradually to 4°C overnight. Competent E. coli Ag1 cells (Stratagene) were transformed with 3 µl of the above ligation reaction and colonies selected using LB/CAM 20 plates. A clone having the appropriate Nhe I/Xho I and Nhe I/Xho I restriction patterns was selected for DNA sequencing.

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The oligonucleotide used to verify the sequence of the FLAG™ linker in pATDFLAG was called PENPTSEQ: 5'-CTTTATGTAAGATGATGTTTTG-3. This oligonucleotide is derived from the non-coding strand of the penP terminator region (See Figure 23). Figure 23 gives the amino acid and DNA sequence in pATDFLAG. The amino acid sequence

5   corresponding to the FLAG™ peptide is given in bold italics. The FLAG™ peptide is out of frame relative to the H4V$_L$-linker sequence which ends at the Xho I site. The DNA sequencing primer, PENPTSEQ, is shown in bold. Nucleotides 824-478 inclusive were verified using the PENPTSEQ primer. At position 624, a 'T' nucleotide present in the original H4V$_L$ sequence has been changed to a 'C' nucleotide resulting in a Val to Ala amino acid residue 89 substitution. These

10   differences are indicated in shadow. The CDR amino acid sequences of H$_4$VL are shown in bold, while the UNIHOPE linker sequence is in bold and underlined. DNA sequencing was performed using the Sequenase™ sequencing kit (U.S. Biochemical, Cleveland, OH) following the manufacturer's directions.

c) Generation of pSC49FLAG

15   The plasmid pATDFLAG (approximately 5 μg, purified from a 2.5 mL culture by the Magic Miniprep system (Promega) was treated at 37°C for 1 h as indicated in the protocol given below:

   DNA - 17 μL

   10X buffer (New England Biolabs #2) - 6 μL

20   Xho I (New England Biolabs) - 2.5 μL (25 units)

   Nhe I (New England Biolabs) - 3.5 μL (17.5 units)

   H$_2$O - 31 μL

The resulting vector fragment was purified from a 4 percent PAG, electroeluted and ethanol precipitated. The obtained DNA was dissolved in 20 μL water.

25   The CC49 V$_H$ insert DNA was obtained from a PCR amplification protocol (Perkin Elmer Cetus) as instructed by the manufacturer. The target DNA (uncut) containing the CC49 V$_H$ was pSCFV UHM 8.1 (Example 1) (0.5 mg). The 5' oligo used for the PCR (100 pmol) was UNI-3 (Example 1). The 3' oligo used (100 pmol) was called SC49FLAG, a 33-mer as follows:

   Nhe I

30   5'-TATTGCTAGCTGA GGA GAC GGT GAC TGA GGT TC-3'.

The resulting amplified DNA was purified from a 4 percent PAG, dissolved in 20 μL water, and then treated at 37°C for 1 h as indicated in the protocol given below:

   DNA - 10 μL (~2 μg)

   10X buffer (New England Biolabs #2) - 6 μL

35   Xho I (New England Biolabs) - 2.5 μL (25 units)

   Nhe I (New England Biolabs) - 3.5 μL (17.5 units)

   water - 38 μL

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The resulting insert fragment was purified and dissolved in 20 µL water. The vector and insert fragments from above were set up in a ligation reaction at 18°C going down to 4°C overnight as follows:

> DNA vector (pATDFLAG-Xho I/Nhe I) - 1.5 µL
>
> DNA insert (PCR amplified CC49 V$_H$-Xho I/Nhe I)-0.8 µL
>
> 10X ligation buffer (Stratagene) - 2 µL
>
> 10 mM ATP - 2 µL
>
> T4 DNA ligase - 1 µL
>
> water - 12.7 µL

Competent E. coli AG1 cells (Stratagene) were transformed using the ligation reaction from above, as directed by the manufacturer. Clone number 1, was selected for further work, and for verification of the constructed DNA sequence. The oligos used as primers for the DNA sequencing were as follows:

> 1) PENPR1, a 21-mer: 5'-AAC ACT GTA GGG ATA GTG GAA-3';
>
> 2) PENPR2, a 20-mer: 5'-GTC TCC CTC CGT TTG AAT AT-3';
>
> 3) TAGVLFR2, a 20-mer: 5'-TGG TAC CAG CAG AAA CCA GG-3;
>
> 4) UHVHSEQ, a 22-mer: 5'-GAT GCT GCG AAG AAG GAT GAC G-3';
>
> 5) TAGVHCDR2, a 21-mer: 5'-ATGG ATT GGA TAT TTT TCT CC-3' and;
>
> 6) TAG VH FR4, a 21-mer: 5'-ACT GGG GTC AAG GAA CCT CAG-3'.

The DNA and amino acid sequence of pSC49FLAG is given in Figure 24. Asp = 1 indicates the first amino acid from H4V$_L$ of the mature single chain antibody species. The UNIHOPE linker is in bold and underlined. The complementarity determining region sequences are shown in bold, while the FLAG peptide is in bold italics. Nucleotides corresponding to the Nhe I site in pSCFV UHH are in bold and underlined (position 1154-1159). The 'C' at position 1155 and the 'A' at position 1157 are italicized within this sequence and indicate differences from the published penP terminator sequence. The DNA sequencing was carried out using the Sequenase™ kit mentioned above.

The predicted pI and molecular weight (MW) of each of the protein species produced was obtained from the DNASTAR™ Protein-Titrate computer program (Madison, WI).

Purification of H4L49HF Single Chain Products

A 10 mL M2 antibody affinity column was prepared (gel purchased from IBI) and incorporated on the HPLC system described under general experimental procedures. The H4L49HF crude protein sample was prepared as follows. One liter (2 X 500 mL) of E. coli AG1/pSC49FLAG was grown overnight at 37°C in LB broth containing 20 µg/mL chloramphenicol. The cells were recovered by centrifugation of the culture at 5,000 rpm for 10 min at 5°C in a Sorvall GS-3 rotor (duPont). The cell pellet was resuspended in 100 mL of PBS, and subjected to sonication (MSE Soniprep 150) for 5 bursts at 14 microns amplitude lasting for 15 seconds each, and cooling in an ethanol-ice bath for 1 minute in between. The sonicate was

ultracentrifuged at 26,000 rpm for 2 hours in a Type Ti30 rotor (Beckman Instruments). Th resulting supernatant was filtered through a 0.22 membrane device (Nalge Co., Rochester, NY) and finally concentrated to 22 mL using Centriprep 30 (Amicon) devices. The sonicate was pumped onto the affinity column at 2 mL/min with stops lasting for 1 min twice during the

5   application, to allow interaction with the affinity ligand.

PBS was used to wash the column of any unbound material, and when the absorbance at 276 nm returned substantially to the baseline, the buffer was changed to 0.1M glycine-HCl, at pH 3.1. Elution of a peak began 4 minutes later, the fractions of which were immediately neutralized using 1 M Tris pH 8.8 and PANPEHA indicator (range 0-14) pH paper

10   strips (Schleicher Schull). The eluted protein was concentrated to 200 µL using Centriprep 30 and Centricon 30 (Amicon) devices.

The resulting single chain antibody species were separated by gel filtration using a Pharmacia Superdex 75 Prep Grade HR 26/60 column and PBS as the eluent. The flow rate was initially 0.4 mL/min, and then increased to 1.2 mL/min during the chromatography. Three pools

15   of fractions from this run were made, and then tested analytically on a Superdex 75 HR10/30 column for purity and characteristic elution time. Biorad gel filtration standards were also chromatographed on this column using the same buffer (PBS) and a 0.6 mL/min flow rate, to generate a standard curve for comparison with the elution times of the single chain antib dy species. The three species obtained were concentrated, quantitated at OD 280 nm and stored

20   frozen at -20°C.

The results of the purification and characterization of the H4L49HF single chain products is given in Figure 25. Panel A shows the M2 affinity chromatography profile for *E. coli* pSC49FLAG sonicate preparation. Stars (*) indicate points in the application of sample when the flow was stopped for 30 seconds. The arrow indicates the point at which the buffer was

25   changed and the flow chart increased to 2.0 mL/min. Fractions were 2 minutes long.

Panel B represents an analytical gel filtration chromatogram obtained from application of 0.5 percent of the total pooled sample from fractions 19-21 shown in A.

Panel C shows a 10-20 percent gradient SDS-PAGE of samples stained with Coomassie Brilliant Blue R-250 (Lanes 1-6) or Western transferred (lanes 7-11) and probed with

30   biotinylated FAID14. Lane 1: prestained molecular weight standards (Diversified Biotech); · lanes 2-4 and 7-9 are purified H4L49HF scFv, Fv2 and Fv4, respectively; lanes 5 and 10; *E. coli* pSC49FLAG sonicate sample, the same as that as applied to M2 affinity column; and lanes 6 and 11, *Pichia* CC49scFv 5.2.

Amino Terminal Sequencing

35   Samples of the three protein species of H4L49HF (10 µg each) were desalted by 3 successive water washings using Centricon 30 devices and freeze-dried. Amino terminal sequencing was determin d by standard Edman degradation techniques.

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Determination of Anti TAG-72 Activity

  a) anti-FLAG ELISA

A. Tag Elisa

Binding activities of the purified monomer and multimer forms of H4L49HF as
well as sonicates of *E. coli* expressing these species were determined by ELISA (Enzyme Linked
Immunosorbant Assay) on 96 well PVC plates coated with a 1/300 dilution of the TAG-72 prep
(040191). The plates were prepared and blocked for two hours with 1 percent bovine serum
albumin (BSA) in PBS with 0.025 percent sodium azide at 37°C. After blocking, the plates were
washed three times with 0.025 percent Tween 20 solution and stored at 4°C.

Aliquots of 50 µL of sample at each concentration were added in duplicate to the
96 well plate. The starting concentrations for the purified monomer, dimer, and tetramer were
10 µg/mL for each, and 1:2 dilutions were made to a final concentration of 0.005 µg/mL. The
samples added to the plate included the purified monomer, dimer, tetramer, and various crude
sonicates.

After adding the samples, the plate went through a series of incubations at 37°C.
The first incubation was for 1 hour with sample. The plate was washed five times with
0.025 percent Tween 20. Binding of FLAG™ containing single chain species was detected by
subsequent incubations with murine anti-FLAG MAb, M2 (International Biotechnologies
cat: IB13010) at 1/250 dilution. The plate was incubated for 1 hour removed, and washed.
Alkaline phosphatase conjugated goat anti-mouse polyclonal antibody (GAMIG-ALPH,
Southern Biotechnology Associates) was added at 50 µL per well. The plate was incubated for
1 hour, removed, and washed. The diluent for both antibodies was in 1 percent BSA in PBS with
0.025 percent sodium azide. The phosphatase substrate (Kirkegaard & Perry Laboratories, Inc.)
was prepared by mixing 1 ml of 5X buffer (Kirkegaard & Perry Kit), 4 mL water, and one
substrate tablet (Kirkegaard & Perry Kit). To each well, 50 µL were added. Color development
was terminated after 15 minutes with 50 µL/well of 1 M sodium hydroxide. The ELISA plate was
read with the Molecular Devices Kinetic Microplate Reader using the Delta Soft© Computer
program.

B. BSM ELISA

Bovine submaxillary mucin (BSM) was applied to a PVC plate at concentrations
ranging from 5.0 mg/ml to 0.00008 mg/ml decreasing at 1:2 dilutions in water. A blank of 50 µL
of PBS with 0.025 percent azide was added as a control. The plate was allowed to dry at room
temperature overnight in a biological safety cabinet. The plate was blocked using 200 µL of
1 percent BSA in PBS. The plate was incubated at 37°C for 50 minutes. The plate was removed
from incubation and washed three times with 0.025 percent Tween 20. Dilutions (1/3, 1/9, 1/27)
of a crude sonicate of pSC49FLAG were applied to the BSM control wells.

After obtaining results from the above ELISA, a BSM concentration of 60 µg/ml
was selected for further plating. The diluent was water. The BSM at 60 µg/ml was loaded at

-55-

50 µL per well using a multi-channel pipet and dried and blocked as above. The plates were washed three times with 0.025 percent Tween 20 and stored at 4°C. Incubation and detection of FLAG-containing species was performed as above, using the M2 anti-FLAG antibody.

The relative binding of H4L49HF single chain species by TAG of BSM ELISA are

5   given in Figure 26. Figure 26A shows results from TAG ELISA for purified monomer, dimer and tetramer. Figure 26B shows results from TAG ELISA for *E. coli* pSC49FLAG sonicate containing monomer and multimer species. Figure 26C shows results from various concentrations of the single chain species reacting with BSM. Figure 26D is ELISA on BSM with pSC49FLAG sonicate.

In Figure 26A, starting with concentrations of 10 ug/ml, 1:3 dilutions were

10   performed and the relative binding to TAG-coated plates was determined. It can be seen that the tetramer species gave the greatest binding, followed by dimer, and eventually by monomer. Using DeltaSoft software (BioMetallics, Princeton, NJ), and assuming the dimer as the standard curve, the tetramer activity was 1.3 to 5 times greater than the dimer activity. The monomer activity was only 0.5 to 0.8 percent of the dimer activity.

15   b) Competition ELISA

This assay was performed as described in Example 1. The formula used to calculate percent competition is as follows:

$$\frac{No\ Competition\ -\ Sample\ reading}{No\ Competition\ -\ Background}\ \text{X}\ 100$$

20
Absorbance values for the samples were measured at 405 nm - 450 nm. The average of duplicate readings was used. Initially samples (25 µL) were applied to the TAG-72 coated microliter plates at 2.0×10^{-10}/mL. Biotin CC49 (4 µg/µL diluted 1:20,000 - used 25 µL) diluted the samples by a factor of 2. Serial dilutions (1:2) were performed, resulting in a total of 10

25   concentrations for each sample. The "no competition" sample consisted of 25 µL of antibody buffer (1 percent BSA in Tris buffered saline (Pierce Chemical Co.) + 0.025 percent Tween and 25 µL of the biotinylated CC49 competitor. Background samples were formulated with either antibody buffer with no added CC49 biotin, or a 5 µg/mL solution of CC49 IgG in antibody buffer. Both give essentially the background reading.

30   The results of the competition ELISA are given in Figures 27 and 28, Figure 27 based on moles of antibody binding site and Figure 28 based on moles of antibody. These results show that the extra FLAG™ binding sites on the dimer and tetramer did not skew the results in favor of the multimers.

Immunohistochemistry (IHC)

35   Specimens. A total of 7 specimens of colorectal adenocarcinoma, including four primary adenocarcinomas and three intra-abdominal metastases (two mesenteric metastases and one hepatic metastasis), were used in these studies. Areas of reactive or normal tissue were also present for assessment of immunoreactivity of non-neoplastic tissues. Sequential 5 µm

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thick sections were prepared. One section was stained with hematoxylin and eosin (H and E) with other sections used for the various IHC procedures.

The purified H4L49HF Fv2 product was used at 0.33, 1.7 and 8.3 µg/mL levels as the primary probing agent for the specimens. Sonication (as per Example 1) of a 2.5 mL

5 overnight culture of E. coli pSC49FLAG provided a crude test probe material and was used for the specimens. These sonicates contained a mixture of H4L49HF scFv, Fv2, Fv4 etc. whose concentration and composition was not known.

Immunohistochemistry procedures (IHC). IHC was performed using a modification of the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., J. Histochem.

10 Cytochem., 29, 577-580, 1981). The method utilized a commercial anti-mouse IgG kit (Vectastain Peroxidase Mouse IgG, Vector Laboratories, Burlingame, CA). Briefly, the technique was as follows:

1) Sequential 5-6 µ sections of the test tumors were deparaffinized, rehydrated, and endogenous peroxidase activity was quenched.

15 The sections were then sequentially flooded with the following reagents:

2) Normal equine blocking serum (Vectastain kit) for 20 minutes.

3) Add primary antibody for 45 minutes, either 200 µL of H4L49HF Fv2 or sonicate of E. coli pSC49FLAG (from 2.5 mL of overnight cell growth), and add to sequential sections of the tumor. Diluent (PBS containing 0.1 percent BSA, Sigma Chemicals Co. St. Louis, MO) was

20 used on one section from every tumor at this step to serve as a negative control.

4) M-2 (International Biotechnologies Inc., San Pedro, CA), murine anti-FLAG MAb, was then used at a dilution of 1:100 for 45 minutes.

5) Secondary antibody, biotinylated anti-mouse IgG (Vectastain kit), for 45 minutes.

25 6) ABC reagent for 45 minutes.

7) The chromagen, 0.05 percent diaminobenzidine, in 0.02 percent H_2O_2 for 3.5 minutes.

8) The slides were counterstained with Meyer's hematoxylin (Sigma Diagnostics, St. Louis, MO), dehydrated, and coverslipped by routine methods.

30 All reactions were conducted at room temperature. The slides were rinsed between each step, except following step 2, in three washes of PBS for at least five minutes each; the final rinse contained 0.1 percent BSA. IHC reactivity was evaluated for both the acellular and cellular parts of the tumor as well as for adjacent non-neoplastic structures

The H4L49HF purified dimer stained both cellular and acellular areas of the test

35 colon carcinomas. Mucinous acellular material from the neoplastic cells was extensively reactive. Viable neoplastic cells had more variable reactivity with some tumors having high numbers of reactive cells and other tumors having few reactive cells. The specific cellular location of the staining was also variable within and between the different specimens. The

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atypical cytoplasm and/or (atypical) surface adjacent stained acellular material was most frequently stained. Lesser members of cells had diffuse cytoplasmic staining. There was little reactivity of H4L49H Fv2 with limited numbers of non-neoplasmic tissues examined except reactive colon mucosa immediately adjacent CRC. In this mucosa, the mucin vacuoles of goblet

5   cells and the free mucin on the surface was reactive. The staining diminished with the distance from the tumor/reactive mucosa junction. Crude *E. coli* pSC49FLAG sonicate, containing all the H4L49HF scFv species (monomer, dimer, multimer, etc.) also gave similar results, indicating that these single chain antibodies are specific for tumor tissues. A control sonicate from *E. coli* pATDFLAG gave no staining of the TAG-72 positive areas.

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Claims:

1.   A protein comprising a dimer or multimer of single chain polypeptides wherein the polypeptides are non-covalently linked and each polypeptide comprises

(a) a first polypeptide comprising an antigen binding portion of a variable
5   domain of an antibody;

(b) a second polypeptide comprising an antigen binding portion of a variable domain of an antibody; and

(c) a peptide linker linking the first and second polypeptides (a) and (b) into a single chain polypeptide having affinity for an antigen.

10   2.   The protein of Claim 1 wherein the first polypeptide is an antigen binding portion of a heavy chain variable region of an antibody and the second polypeptide is an antigen binding portion of a light chain variable region of an antibody.

3.   The protein of Claim 1 wherein the first polypeptide is an antigen binding portion of a light chain variable region of an antibody and the second polypeptide is an
15   antigen binding portion of a heavy chain variable region of an antibody.

4.   The protein of Claim 3 wherein the polypeptide is a dimer.

5.   The protein of any one of Claims 1 to 4 conjugated to an imaging marker.

6.   The protein of Claim 5 wherein the imaging marker is ^{125}I, ^{131}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re or ^{99m}Tc.

20   7.   A composition comprising a protein of any one of Claims 1 to 6 in a pharmaceutically acceptable carrier.

8.   The protein of any one of Claims 1 to 4 conjugated to a therapeutic agent.

9.   The protein of Claim 8 wherein the therapeutic agent is a radionuclide, drug or biological response modifier or toxin.

25   10.   The composition of Claim 9 wherein the radionuclide is ^{131}I, ^{90}Y, ^{105}Rh, ^{47}Sc, ^{67}Cu, ^{212}Bi, ^{211}At, ^{67}Ga, ^{125}I, ^{186}Re, ^{188}Re, ^{177}Lu, ^{99m}Tc, ^{153}Sm, ^{123}I or ^{111}In.

11.   A composition of any one of Claims 8 to 10 in a pharmaceutically acceptable carrier.

12.   A method of imaging the internal structure of an animal comprising
30   administering to the animal an effective amount of a composition of Claim 6 and measuring detectable radiation associated with the animal.

13.   A method for producing a dimer or multimer of single chain polypeptides wherein the polypeptides are non-covalently linked and each polypeptide comprises (a) a first polypeptide comprising an antigen binding portion of a variable domain of an antibody; (b) a
35   second polypeptide comprising an antigen binding portion of a variable domain of an antibody; and (c) a peptide linker linking the first and second polypeptides (a) and (b) into a single chain polypeptide having affinity of an antigen, the method comprising:

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(i)   providing a genetic sequence coding for a
single chain polypeptide;

(ii)   transforming a host cell with the genetic
sequence;

5   (iii)   expressing the genetic sequence in the
host cell; and

(iv)   recovery of single chain polypeptides
which have non-covalently linked to form
dimers and multimers.

10   14.   The method of Claim 13 wherein genetic sequence coding for a single
chain polypeptide contains a sequence encoding for a signal peptide which will direct secretion
of the single chain polypeptide out of the cytoplasm of the host cell.

15.   The protein of any one of Claims 1-4 wherein the linker is encoded by a
nucleotide sequence of

15   5'-CTTAGTGCGGACGATGCGAAAAAGGATGCTGCGAAGAAGGATGACGCTAAG
AAAGACGATGCTAAAAAGGACCTC-3'.

16.   The protein of any of Claims 1-4 wherein the linker has the amino acid
sequence Leu Ser Ala Asp Asp Ala Lys Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys
Lys Asp Leu.

20   17.   A method of detecting an antigen in or suspected of being in a sample
which comprises:

(a) contacting the sample with the protein of any one of Claims 1 to 4; and

(b) detecting whether the protein has bound to the antigen.
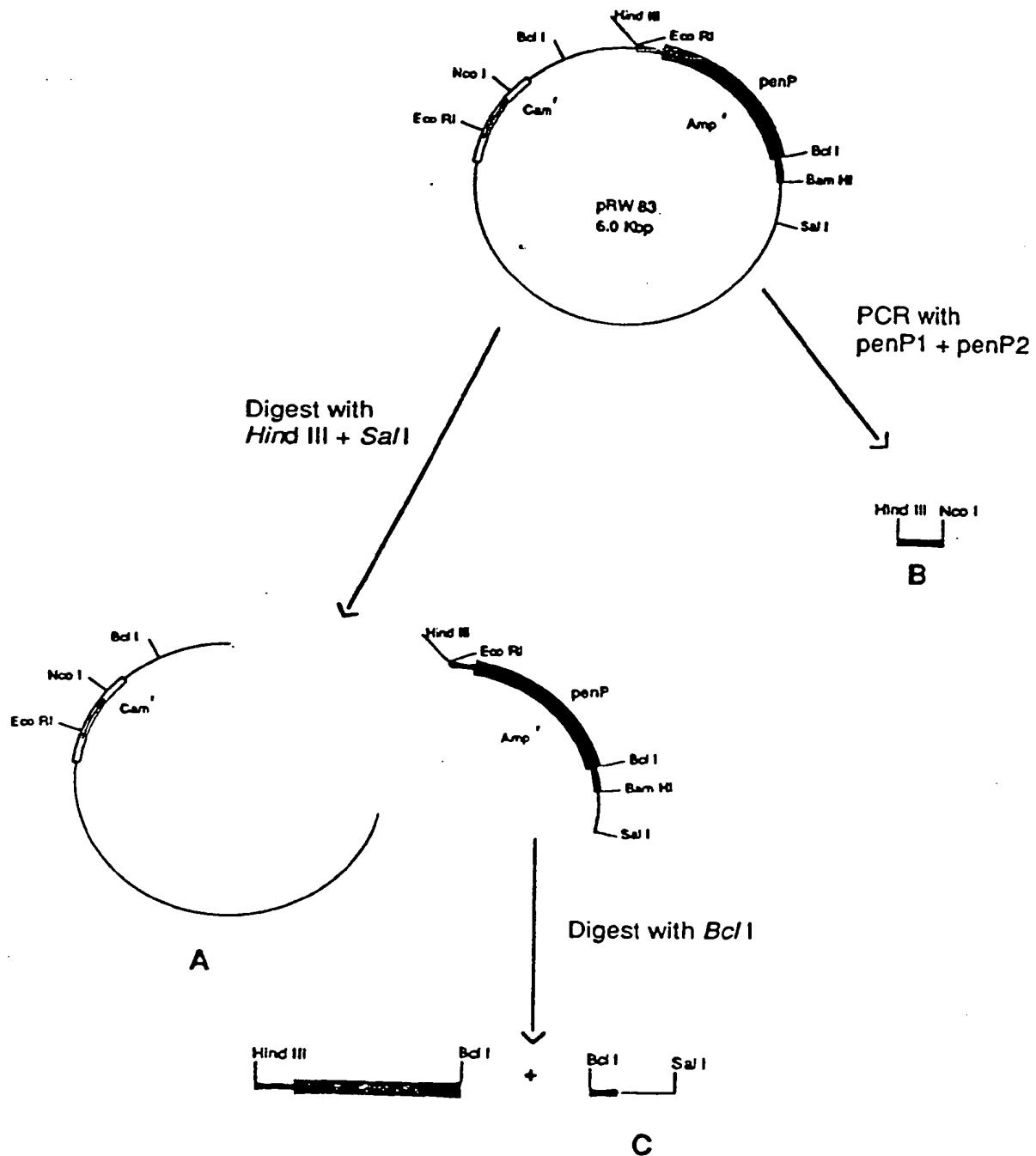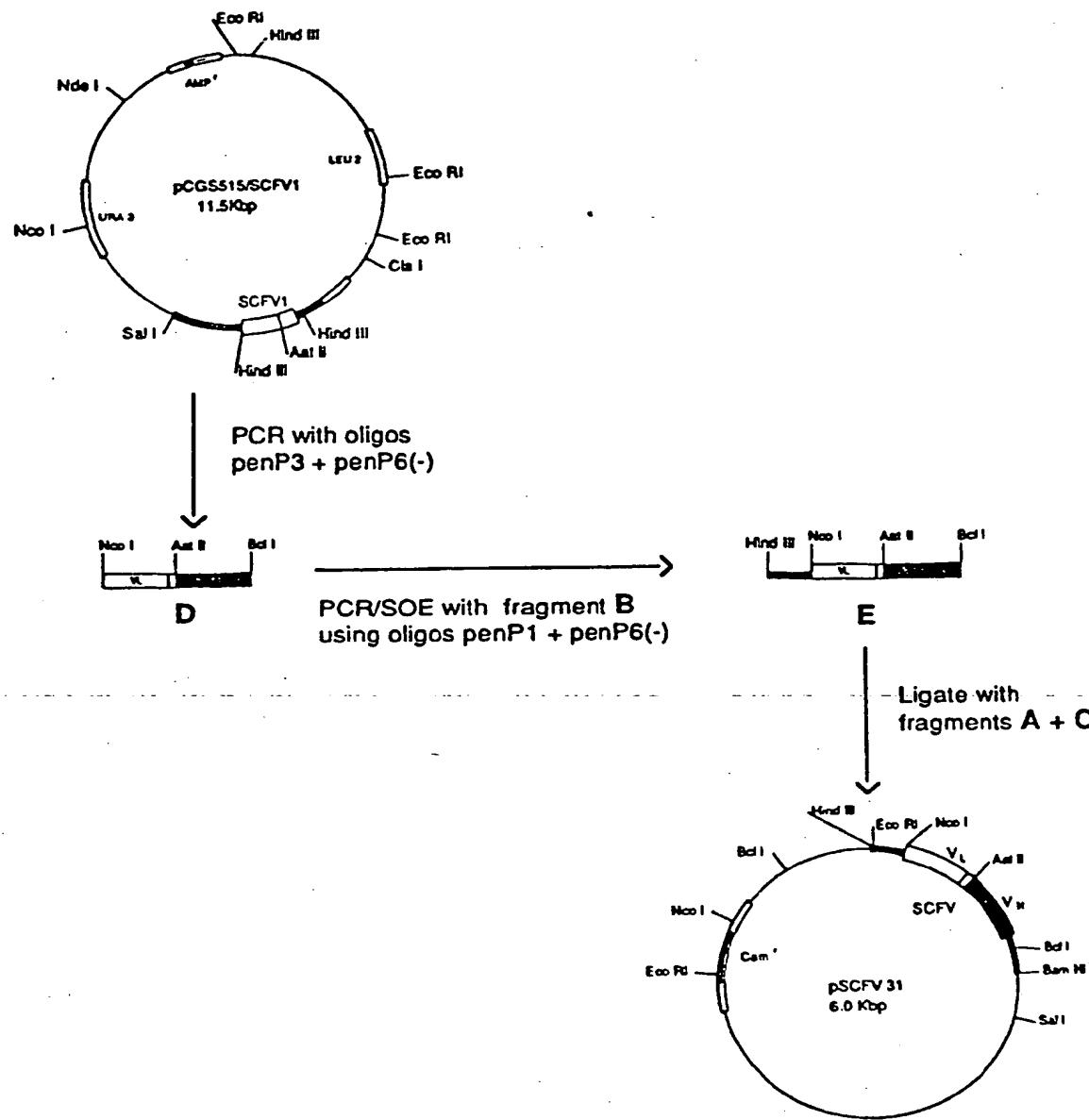
25

30

35

FIGURE 1

CONSTRUCTION OF PLASMID pSCFV31

PCR with
penP1 + penP2

Digest with
Hind III + Sal I

Digest with Bcl I

FIGURE 2

CONSTRUCTION OF PLASMID pSCFV31

FIGURE 3

```
                      Met Leu
AAAAACTAT AAGCTCCATG ATG CTT


Leu Gln Ala Phe Leu Phe Leu Leu Ala
TTG CAA GCT TTC CTT TTC CTT TTG GCT


Gly Phe Ala Ala Lys Ile Ser Ala Asp
GGT TTT GCA GCC AAA ATA TCT GCA GAC


Ile Val Met Thr Gln Ser Pro Asp Ser
ATC GTG ATG ACC CAG TCT CCA GAC TCC


Leu Ala Val Ser Leu Gly Glu Arg Ala
CTG GCT GTG TCT CTG GGC GAG AGG GCC


            :-------------CDR1L--
Thr Ile Asn Cys Lys Ser Ser Gln Ser
ACC ATC AAC TGC AAG TCC AGC TGC AAG


----------------------------------
Val Leu Tyr Ser Ser Asn Asn Lys Asn
GTT TTA TAC AGC TCC AAC AAT AAG AAC


-----------:
Tyr Leu Ala Trp Tyr Gln Gln Lys Pro
TAC TTA GCT TGG TAC CAG CAG AAA CCA


Gly Gln Pro Pro Lys Leu Leu Ile Tyr
GGA CAG CCT CCT AAG CTG CTC ATT TAC


:----------CDR2L-----------:
Trp Ala Ser Thr Arg Glu Ser Gly Val
TGG GCA TCT ACC CGG GAA TCC GGG GTC


Pro Asp Arg Phe Ser Gly Ser Gly Ser
CCT GAC CGA TTC AGT GGC AGC GGG TCT


Gly Thr Asp Phe Thr Leu Thr Ile Ser
GGG ACA GAT TTC ACT CTC ACC ATC AGC


Ser Leu Gln Ala Glu Asp Val Ala Val
AGC CTG CAG GCT GAA GAT GTG GCA GT
```

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FIGURE 3 Continued

```
          :----------CDR3L---------
Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr
TAT TAC TGT CAG CAA TAT TAT AGT TAT


----------:
Pro Leu Thr Phe Gly Gly Gly Thr Lys
CCT CTC ACT TTC GGC GGA GGG ACC AAG


     :----------------------------
Val Lys Glu Ser Gly Ser Val Ser Ser
GTG AAG GAG TCA GGT TCG GTC TCC TCA


-------LINKER--------------------
Glu Gln Leu Ala Gln Phe Arg Ser Leu
GAA CAA TTG GCC CAA TTT CGT TCC TTA


---:
Asp Val Gln Leu Gln Gln Ser Asp Ala
GAC GTC CAG TTG CAG CAG TCT GAC GCT


Glu Leu Val Lys Pro Gly Ala Ser Val
GAG TTG GTG AAA CCT GGG GCT TCA GTG


Lys Ile Ser Cys Lys Ala Ser Gly Tyr
AAG ATT TCC TGC AAG GCT TCT GGC TAC


          :----------CDR1H----:
Thr Phe Thr Asp His Ala Ile His Trp
ACC TTC ACT GAC CAT GCA ATT CAC TGG


Val Lys Gln Asn Pro Glu Gln Gly Leu
GTG AAA CAG AAC CCT GAA CAG GGC CTG


          :--------------------
Glu Trp Ile Gly Tyr Phe Ser Pro Gly
GAA TGG ATT GGA TAT TTT TCT CCC GGA


------------------CDR2H------------
Asn Asp Asp Phe Lys Tyr Asn Glu Arg
AAT GAT GAT TTT AAA TAC AAT GAG AGG


----------:
Phe Lys Gly Lys Ala Thr Leu Thr Ala
TTC AAG GGC AAG GCC ACA CTG ACT GCA
```

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FIGURE 3 Continued

```
Asp Lys Ser Ser Ser Thr Ala Tyr Val
GAC AAA TCC TCC AGC ACT GCC TAC GTG


Gln Leu Asn Ser Leu Thr Ser Glu Asp
CAG CTC AAC AGC CTG ACA TCT GAG GAT


                                :---
Ser Ala Val Tyr Phe Cys Thr Arg Ser
TCT GCA GTG TAT TTC TGT ACA AGA TCC


---CDR3H-----------:
Leu Asn Met Ala Tyr Trp Gly Gln Gly
CTG AAT ATG GCC TAC TGG GGT CAA GGA


Thr Ser Val Thr Val Ser
ACC TCA GTC ACC GTC TCC TAG TGA


AGCTTGGAAC ACCACACAAA CCATATCCAA A
```

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CONSTRUCTION OF *E.COLI* SCFV EXPRESSION PLASMIDS
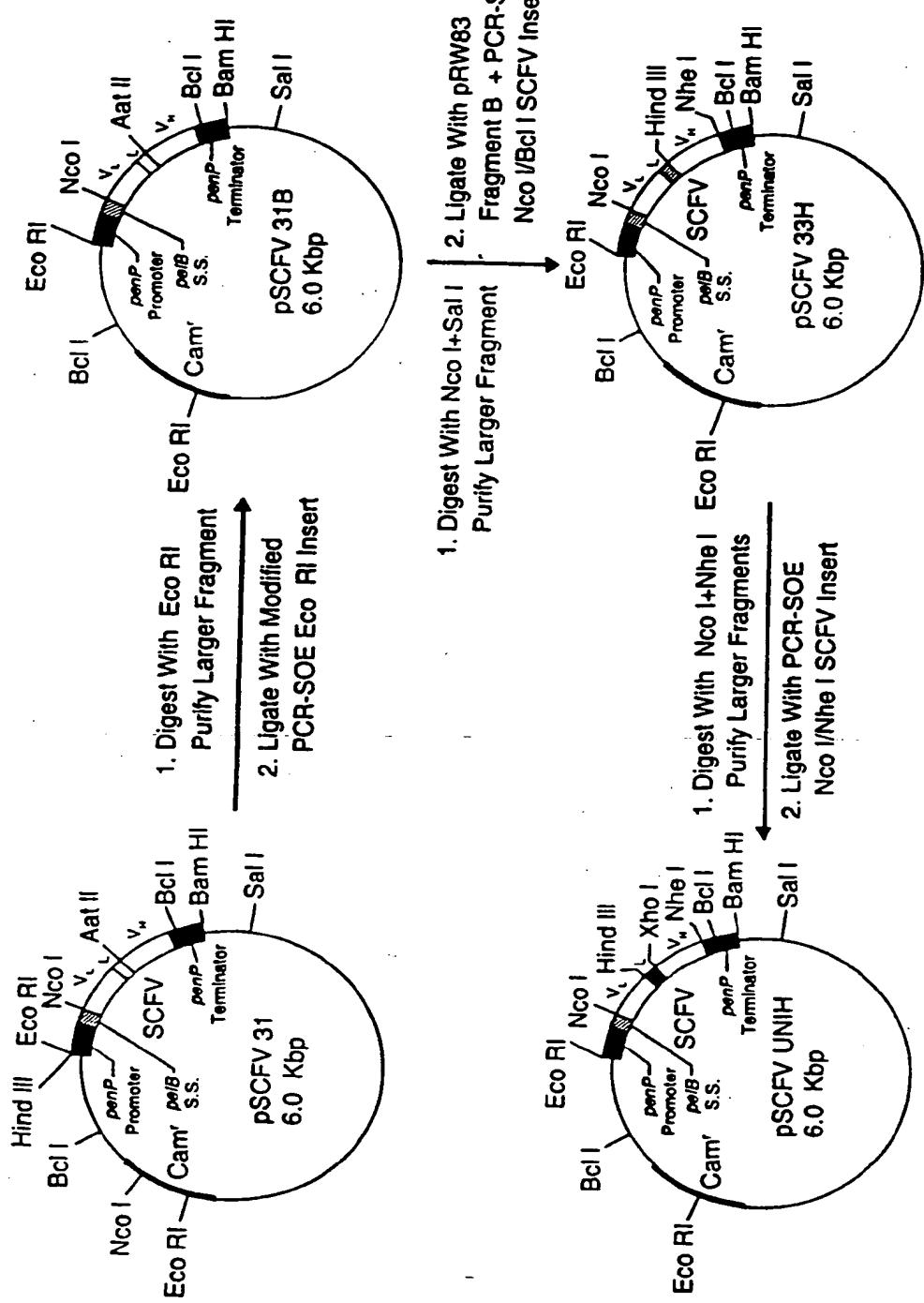CONTAINING HUM V_LIV

FIGURE 4

FIGURE 5


```
     Cla I
3' ATCGATAAAA TTTATTGAGA ATTTGTTTAT TATGATTAAC AGAGGTAAAA GCCAGTATAT    3438
3' TAGCTATTTT AAATAACTCT TAAACAAATA ATACTAATTG TCTCCATTTT CGGTCATATA


TACTGATTAA TATAGGTAAA AGGCAGTTAA GAAATTGGGA ATGCTTTCTC TTCTGCTTTC     3498
ATGACTAATT ATATCCATTT TCCGTCAATT CTTTAACCCT TACGAAAGAG AAGACGAAAG


TTCTACGATG CACAAGGCGT TTCACATTTA TGCCCCTATG AAAATTACTA GGCTGTCCTA     3558
AAGATGCTAC GTGTTCCGCA AAGTGTAAAT ACGGGGATAC TTTTAATGAT CCGACAGGAT


GTCATTAGAT CTTTCAGCAG TTTGTAGTTT TAGAGCTTCT AAGTTGACTT CTGTCTTTTC     3618
CAGTAATCTA GAAAGTCGTC AAACATCAAA ATCTCGAAGA TTCAACTGAA GACAGAAAAG


TATTCATACA ATTACACATT CTGTGATGAT ATTTTTGGCT CTTGATTTAC ATTGGGTACT     3678
ATAAGTATGT TAATGTGTAA GACACTACTA TAAAAACCGA GAACTAAATG TAACCCATGA
        HUMLIN1(-)


TTCACAACCC ACTGCTCATG AAATTTGCTT TTGTACTACT GGTTGTTTTT GCATAGGCCC     3738
AAGTGTTGGG TGACGAGTAC TTTAAACGAA AACATGATGA CCAACAAAAA CGTATCCGGG


CTCCAGGCCA CGACCAGGTG TTTGGATTTT ATAAACGGGC CGTTTGCATT GTGAACTGAG     3798
GAGGTCCGGT GCTGGTCCAC AAACCTAAAA TATTTGCCCG GCAAACGTAA CACTTGACTC


                       Met Val Leu Gln Thr Gln Val Leu Ile
CTACAACAGG CAGGCAGGGG CAGCAAG ATG GTG TTG CAG ACC CAG GTC TTC ATT     3852
GATGTTGTCC GTCCGTCCCC GTCGTTC TAC CAC AAC GTC TGG GTC CAG AAG TAA


Ser Leu Leu Leu Trp Ile Ser G:Intron
TCT CTG TTG CTC TGG ATC TCT G GTGA GGAATTAAAA AGTGCCACAG TCTTTTCAGA   3908
AGA GAC AAC GAG ACC TAG AGA C CACT CCTTAATTTT TCACGGTGTC AGAAAAGTCT
                                                    HUMLIN2(-)


GTAATATCTG TGTAGAAATA AAAAAAATTA AGATATAGTT GGAAATAATG ACTATTTCCA     3968
CATTATAGAC ACATCTTTAT TTTTTTTAAT TCTATATCAA CCTTTATTAC TGATAAAGGT


     Bam HI
ATATGGATCC AATTATCTGC TGACTTATAA TACTACTAGA AAGCAAATTT AAATGACATA     4028
TATACCTAGG TTAATAGACG ACTGAATATT ATGATGATCT TTCGTTTAAA TTTACTGTAT
```


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FIGURE 5 Continued

TTTCAATTAT ATCTGAGACA GCGTGTATAA GTTTATGTAT AATCATTGTC CATTACTGAC   4088
AAAGTTAATA TAGACTCTGT CGCACATATT CAAATACATA TTAGTAACAG GTAATGACTG

                          +1
        ly Ala Tyr Gly Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu
     TACAG GT GCC TAC GGG GAC ATC GTG ATG ACC CAG TCT CCA GAC TCC CTG   4137
     ATGTC CA CGG ATG CCC CTG TAG CAC TAC TGG GTC AGA GGT CTG AGG GAC

                                            :---------------
     Ala Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Lys Ser Ser Gin
     GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC TGC AAG TCC AGC CAG   4185
     CGA CAC AGA GAC CCG CTC TCC CGG TGG TAG TTG ACG TTC AGG TCG GTC

     Ser Val Leu Tyr Ser Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr Gln
     AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC CAG   4233
     TCA CAA AAT ATG TCG AGG TTG TTA TTC TTG ATG AAT CGA ACC ATG GTC
                                  HUMLCDR1(-)

                                          :------CDR 2----
     Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr
     CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG GCA TCT ACC   4281
     GTC TTT GGT CCT GTC GGA GGA TTC GAC GAG TAA ATG ACC CGT AGA TGG

     -----------:
     Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr
     CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG TCT GGG ACA   4329
     GCC CTT AGG CCC CAG GGA CTG GCT AAG TCA CCG TCG CCC AGA CCC TGT


     Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp Val Ala Val
     GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT GTG GCA GTT   4377
     CTA AAG TGA GAG TGG TAG TCG TCG GAC GTC CGA CTT CTA CAC CGT CAA

             :---------------CDR 3------------:
     Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Gly Gly
     TAT TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC ACT TTC GGC GGA GGG   4425
     ATA ATG ACA GTC GTT ATA ATA TCA ATA GGA GAG TGA AAG CCG CCT CCC

     Thr Lys Val Val Ile Lys A   Hind III
     ACC AAG GTG GTG ATC AAA C GTAAGTACAC TTTTCTAAG CTT 3' human Cκ
     4466
     TGG TTC CAC CAC TAG TTT G CATTCATGTG AAAAGATTC GAA 5' insert

ANTI TAG-72 SINGLE CHAIN ANTIBODY
EXPRESSION PLASMIDS AND PRODUCTS

FIGURE 6

SINGLE CHAIN Fv MONOMER
(scFv)

SINGLE CHAIN Fv Dimer
(Fv2)
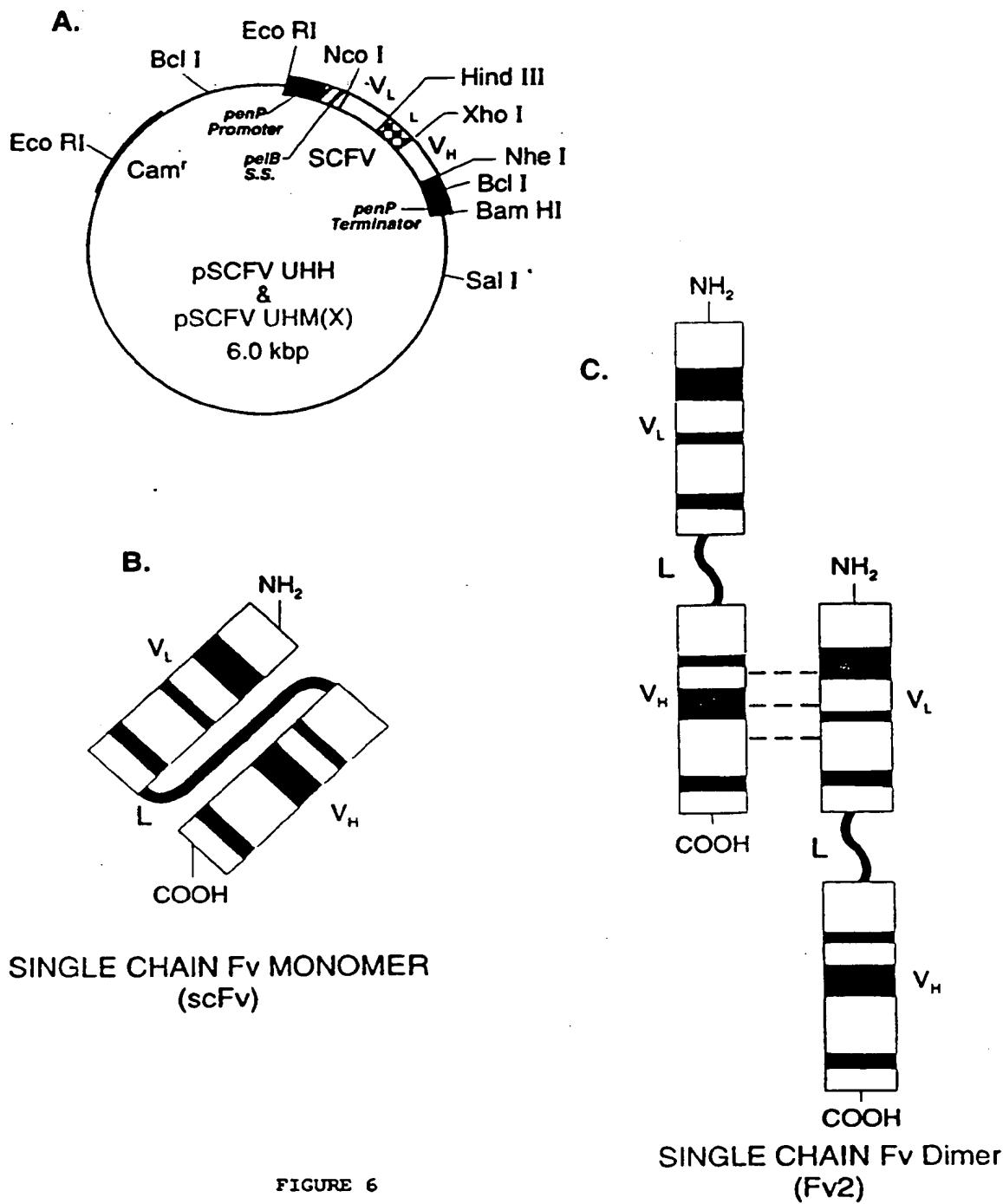
FIGURE 7

DNA AND AMINO ACID SEQUENCES OF H4V$_L$-CC49 V$_H$ SCFV

                                         Cla I   *Eco*R I
5'-C TCA TGT TTG ACA GCT TAT C̲A̲T̲ ̲C̲G̲A̲ ̲T̲G̲A̲ ̲A̲T̲T̲ ̲C̲CA TCA CTT CCC TCC   46

GTT CAT TTG TCC CCG GTG GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA   94

ACG GTT GCA TTT AAA TCT TAC ATA TAT AAT ACT TTC AAA GAC TAC ATT   142

TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT ACG TAC CAA TTA   190

TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA AGA   238

GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG   286

          -22
          Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
ATT TTG ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA   334

                  Nco I   +1
Leu Ala Ala Gln Pro Ala Met Ala Asp Ile Val Met Thr Gln Ser Pro
CTC GCT GCC CAA CCA G̲C̲C̲ ̲A̲T̲G̲ ̲G̲CC GAC ATC GTG ATG ACC CAG TCT CCA   382

     10   20
Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Lys
GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC TGC AAG   430

              30   40
Ser Ser Gln Ser Val Leu Tyr Ser Ser Asn Asn Lys Asn Tyr Leu Ala
TCC AGC CAG AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT   478

                              50
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp
TGG TAC CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG   526

         60   70
Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GCA TCT ACC CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG   574

                         80
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT   622

     90   100
Val Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
GTG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC ACT TTC   670

                 110   *Hind* III   120
Gly Gly Gly Thr Lys Val Val Ile Lys Leu Ser Ala Asp Asp Ala Lys
GGC GGA GGG ACC AAG GTG GTG ATC A̲A̲G̲ ̲C̲T̲T̲ AGT GCG GAC GAT GCG AAA   718

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FIGURE 7 Continued

                                    130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG   766

      Xho I 140   150
Asp Leu Glu Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
GAC CTC GAG GTT CAG TTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT   814

                                160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT   862

      170   180
Asp His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu
GAC CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA   910

                  190   200
Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu
TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG   958

                                210
Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr
AGG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT   1006

      220   230
Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT   1054

                  240
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA   1102

      250
Val Thr Val Ser Ser ***   Nhe I
GTC ACC GTC TCC TCA TAA AAA GCT AGC GAT GAA TCC GTC AAA ACA TCA   1150

TCT TAC ATA AAG TCA CTT GGT GAT CAA GCT CAT ATC ATT GTC CGG CAA   1198

TGG TGT GGG CTT TTT TTG TTT TCT ATC TTT AAA GAT CAT GTG AAG AAA   1246

AAC GGG AAA ATC GGT CTG CGG GAA AGG ACC GGG TTT TTG TCG AAA TCA   1254

                              BamH I
TAG GCG AAT GGG TTG GAT TGT GAC AAA ATT CGG ATC C-3'   1291

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FIGURE 8

DNA AND AMINO ACID SEQUENCES OF CC49 SCFV SPECIES

                                        Cla I   *EcoR I*
5'-C TCA TGT TTG ACA GCT TAT C<u>AT CGA TGA ATT C</u>CA TCA CTT CCC TCC   46

GTT CAT TTG TCC CCG GTG GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA   94

ACG GTT GCA TTT AAA TCT TAC ATA TAT AAT ACT TTC AAA GAC TAC ATT   142

TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT ACG TAC CAA TTA   190

TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA AGA   238

GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG   286

            -22
            Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
ATT TTG ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA   334

                Nco I   +1
Leu Ala Ala Gln Pro Ala Met Ala Asp Ile Val Met Ser Gln Ser Pro
CTC GCT GCC CAA CCA G<u>CC ATG G</u>CC GAC ATT GTG ATG TCA CAG TCT CCA   382

     10   20
Ser Ser Leu Pro Val Ser Val Gly Glu Lys Val Thr Leu Ser Cys **Lys**
TCC TCC CTA CCT GTG TCA GTT GGC GAG AAG GTT ACT TTG AGC TGC AAG   430

            30   40
Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala
TCC AGT CAG AGC CTT TTA TAT AGT GGT AAT CAA AAG AAC TAC TTG GCC   478

                            50
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr **Trp**
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG   526

            60   70
Ala Ser Ala Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly
GCA TCC GCT AGG GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA   574

                        80
Ser Gly Thr Asp Phe Thr Leu Ser Ile Ser Ser Val Lys Thr Glu Asp
TCT GGG ACA GAT TTC ACT CTC TCC ATC AGC AGT GTG AAG ACT GAA GAC   622

        90   100
Leu Ala Val Tyr Tyr Cys Gln Gln **Tyr Tyr Ser Tyr Pro Leu Thr** Phe
CTG GCA GTT TAT TAC TGT CAG CAG TAT TAT AGC TAT CCC CTC ACG TTC   670

                110   *Hind III*   120
Gly Ala Gly Thr Lys Leu Val Leu Lys <u>Leu Ser Ala Asp Asp Ala Lys</u>
GGT GCT GGG ACC AAG CTG GTG CTG <u>AAG CTT</u> AGT GCG GAC GAT GCG AAA   718

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FIGURE 8 Continued

```
                                        130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG    766


        Xho I   140                                          150
Asp Leu Glu Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
GAC CTC GAG GTT CAG TTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT    814


                                160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT    862


        170                                     180
Asp His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu
GAC CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA    910


                        190                                 200
Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu
TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG    956


                                210
Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr
AGG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT    1006


        220                                     230
Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT    1054


                        240
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA    1102


        250
A.  Val Thr Val Ser | Ser ***
        GTC ACC GTC TCC | TCA TAA                                   1120
        250
B.  Val Thr Val Ser | Pro Glu Asp Tyr Asp Ser ***
        GTC ACC GTC TCC | CCT GAG GAC TAT GAC TCC TAA                1135
        250                                     260
C.  Val Thr Val Ser | Pro Glu Asp Pro Glu Asp Tyr Asp ***
        GTC ACC GTC TCC | CCT GAA GAC CCT GAA GAC TAT GAC TAA        1142


        Nhe I
AAA GCT AGC GAT GAA TCC GTC AAA ACA TCA TCT TAC ATA AAG TCA CTT    1189

GGT GAT CAA GCT CAT ATC ATT GTC CGG CAA TGG TGT GGG CTT TTT TTG    1237

TTT TCT ATC TTT AAA GAT CAT GTG AAG AAA AAC GGG AAA ATC GGT CTG    1285

CGG GAA AGG ACC GGG TTT TTG TCG AAA TCA TAG GCG AAT GGG TTG GAT    1333


        BamH I
TGT GAC AAA ATT CGG ATC C-3'                    1354(A); 1369(B); 1375(C)
```

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SDS - PAGE CATION EXCHANGE CHROMATOGRAPHY & ACTIVITY OF
E. COLI/pSCFV UHM 8.1 PERIPLASMIC FRACTION
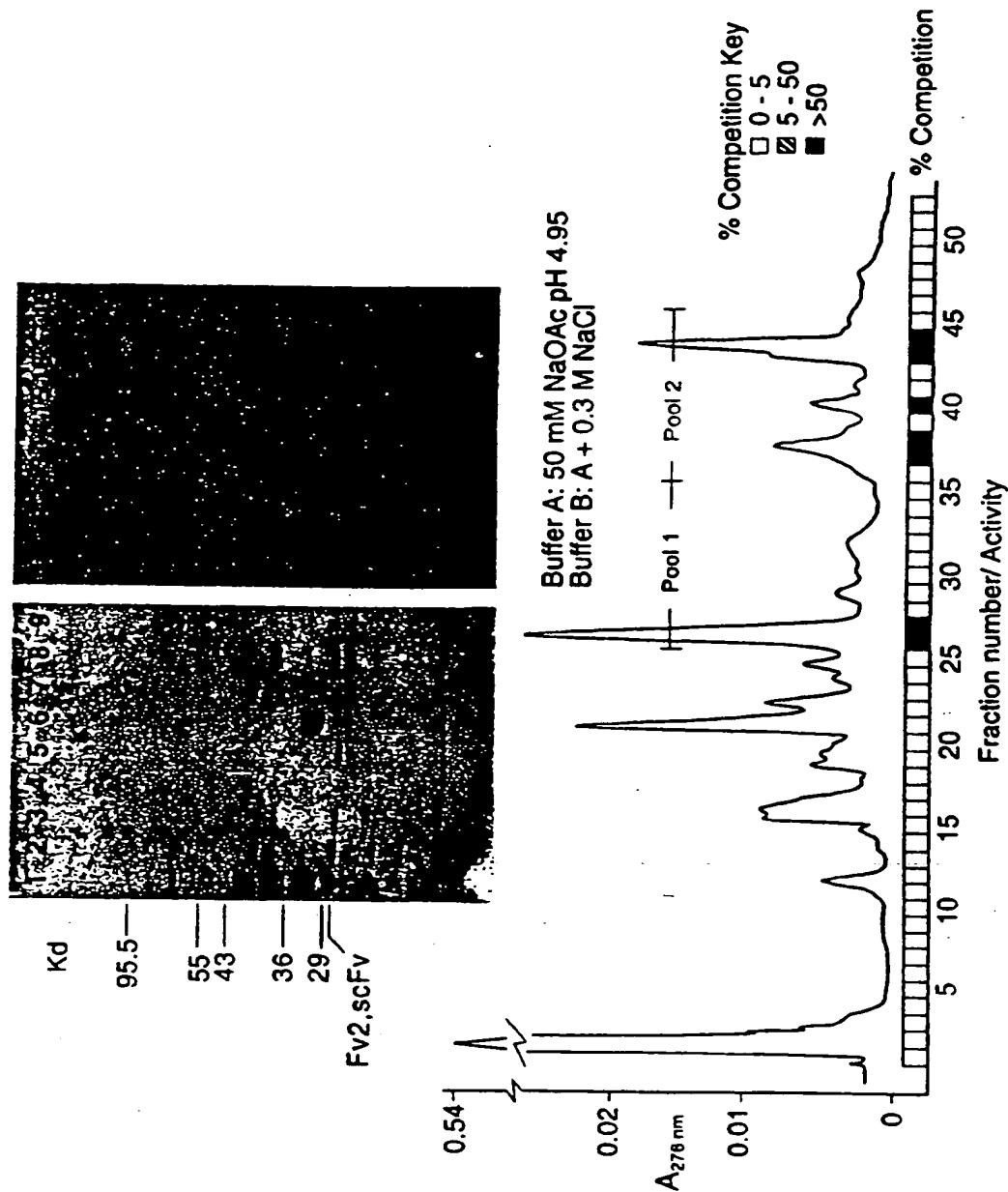
FIGURE 9

ANION EXCHANGE CHROMATOGRAPHY & SDS-PAGE OF MONO S
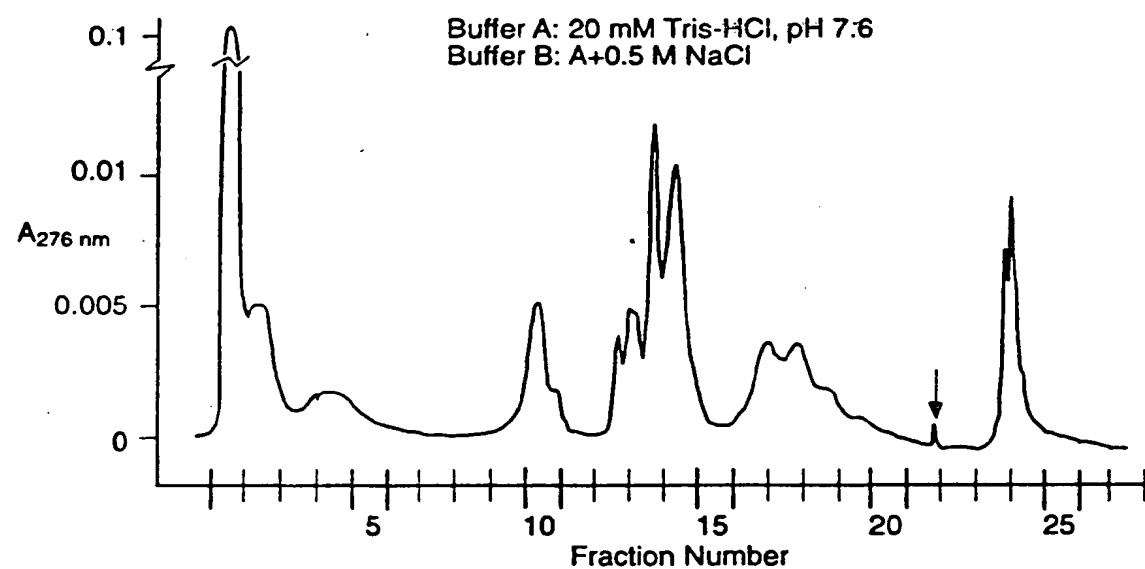## POOL 2 *E.COLI* pSCFV UHM 8.1 PERIPLASMIC FRACTION
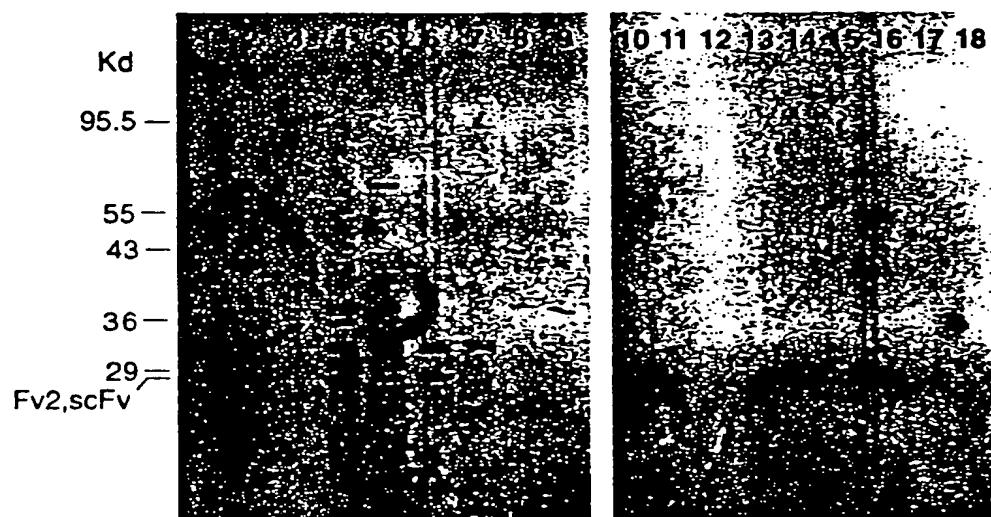
FIGURE 10

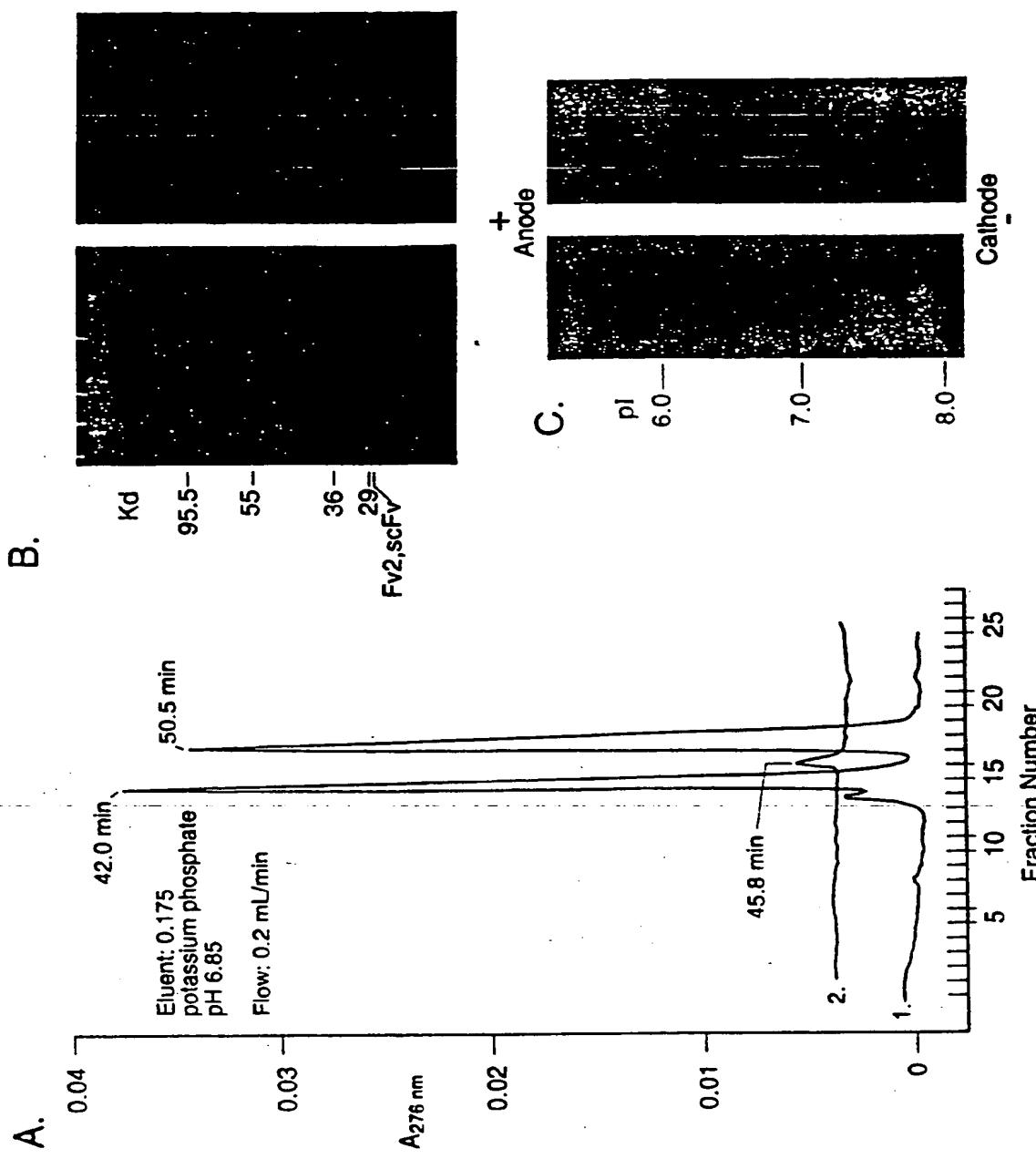GEL FILTRATION, SDS - PAGE & IEF CORRELATION IN THE DISCOVERY OF FV2

A.

B.

C.

FIGURE 11

GEL FILTRATION CHROMATOGRAPHY, ACTIVITY & SDS - PAGE
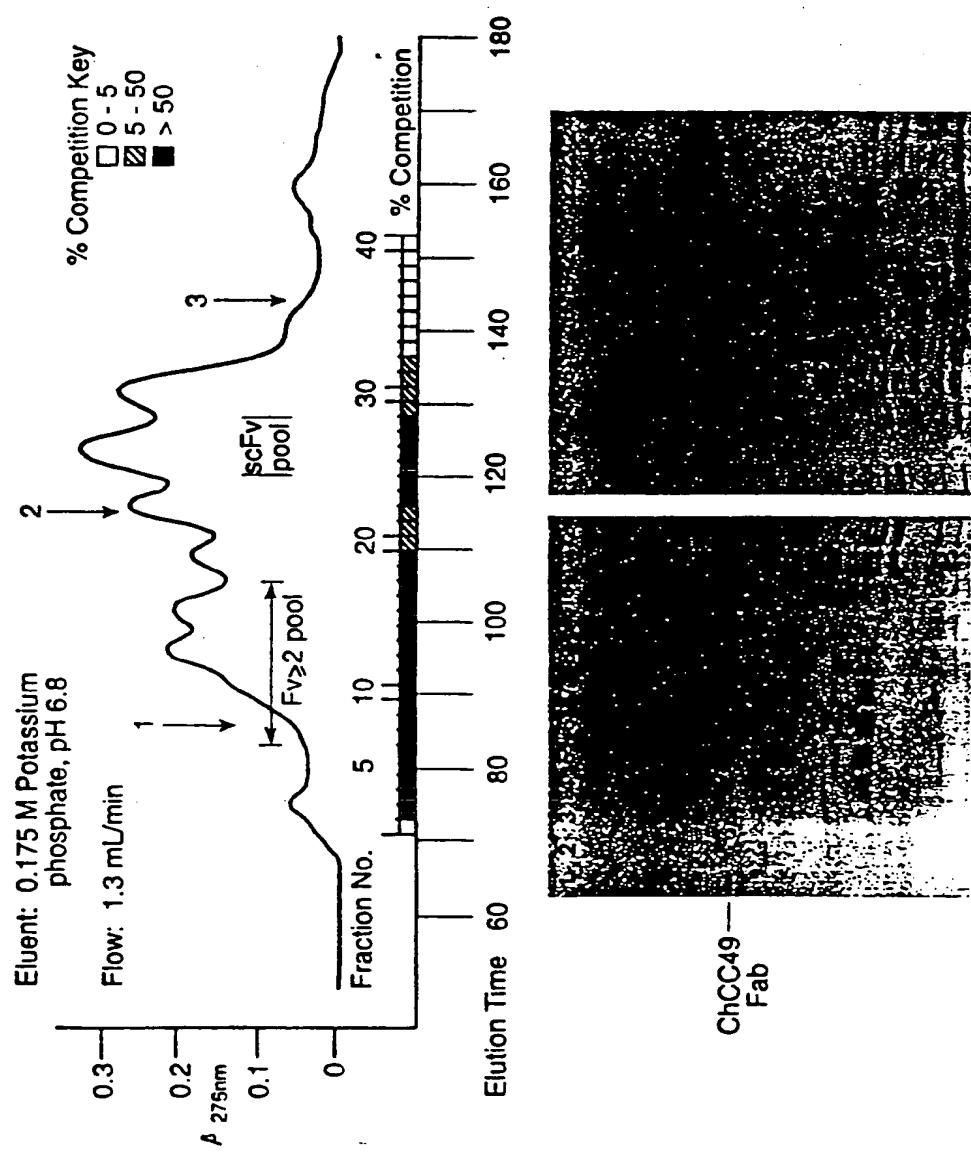## OF *E. COLI* pSCFVUHM 5.2 PERIPLASMIC FRACTION

FIGURE 12

CC49 DIMER/MONOMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG

MOLES OF ANTIBODY BINDING SITES $(\times 10^{-19})$

CC49 IgG   CC49 Fv2-8.1   CC49 scFv8.1

FIGURE 13   (Panel 5)

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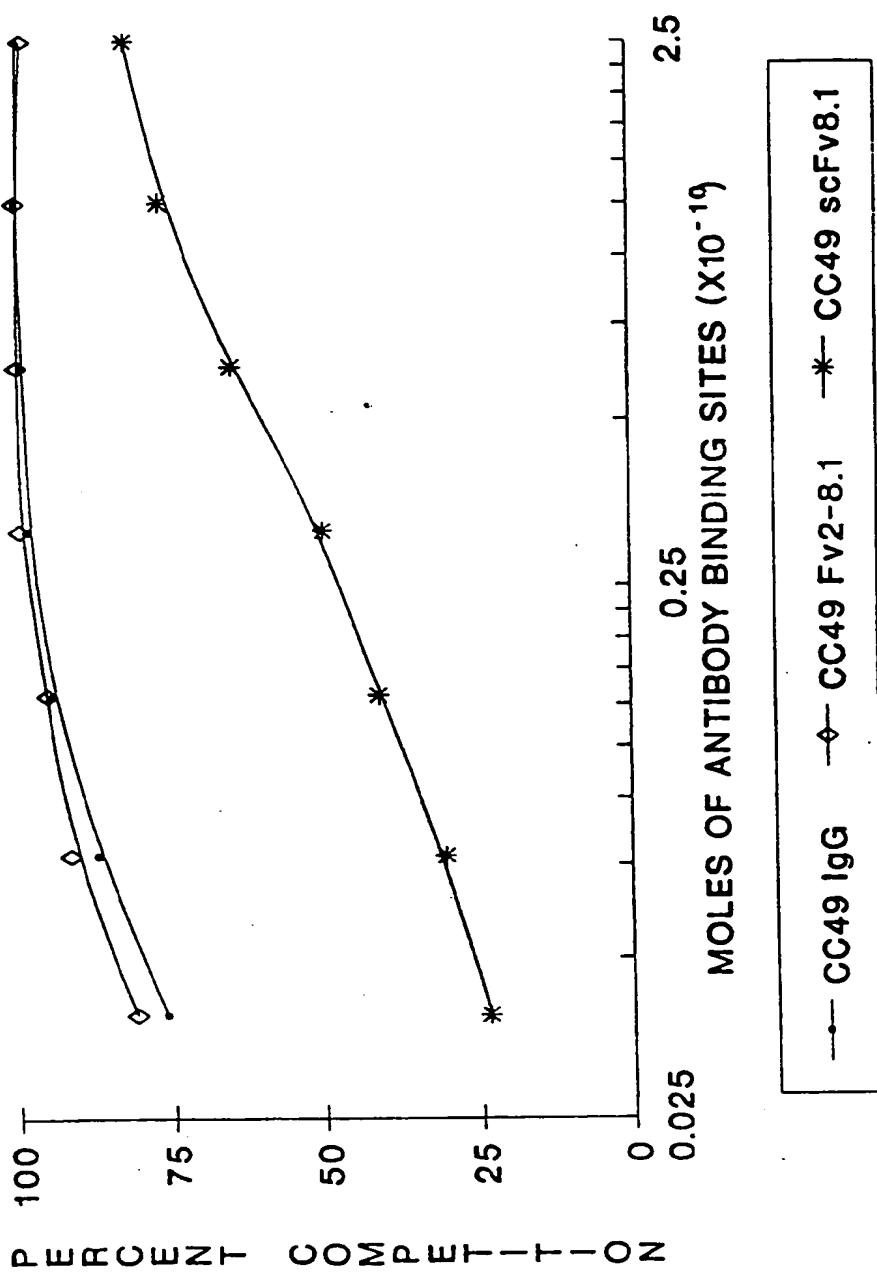
CC49 MONOMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG
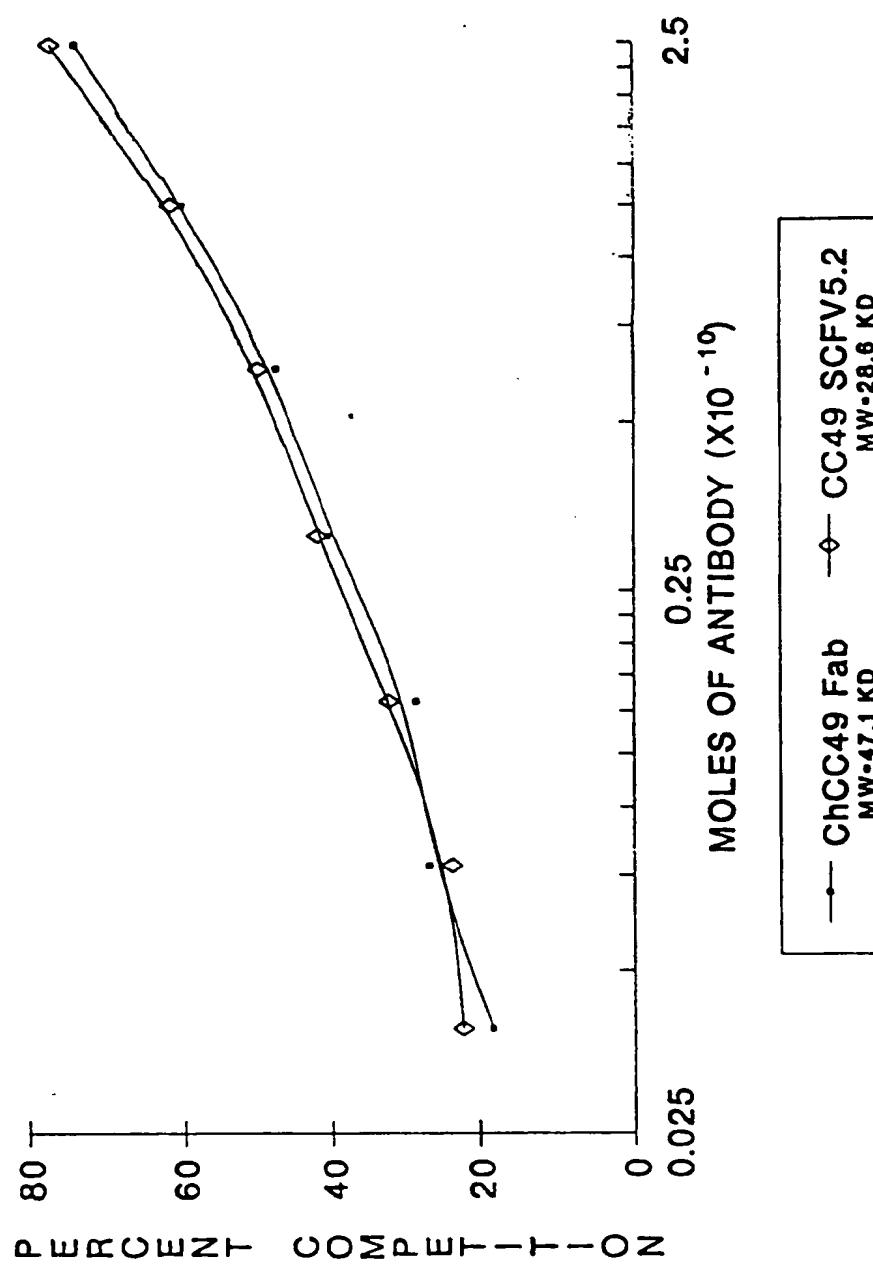
FIGURE 14

PLASMID CONSTRUCTIONS LEADING TO pPY31& pPY32

FIGURE 15

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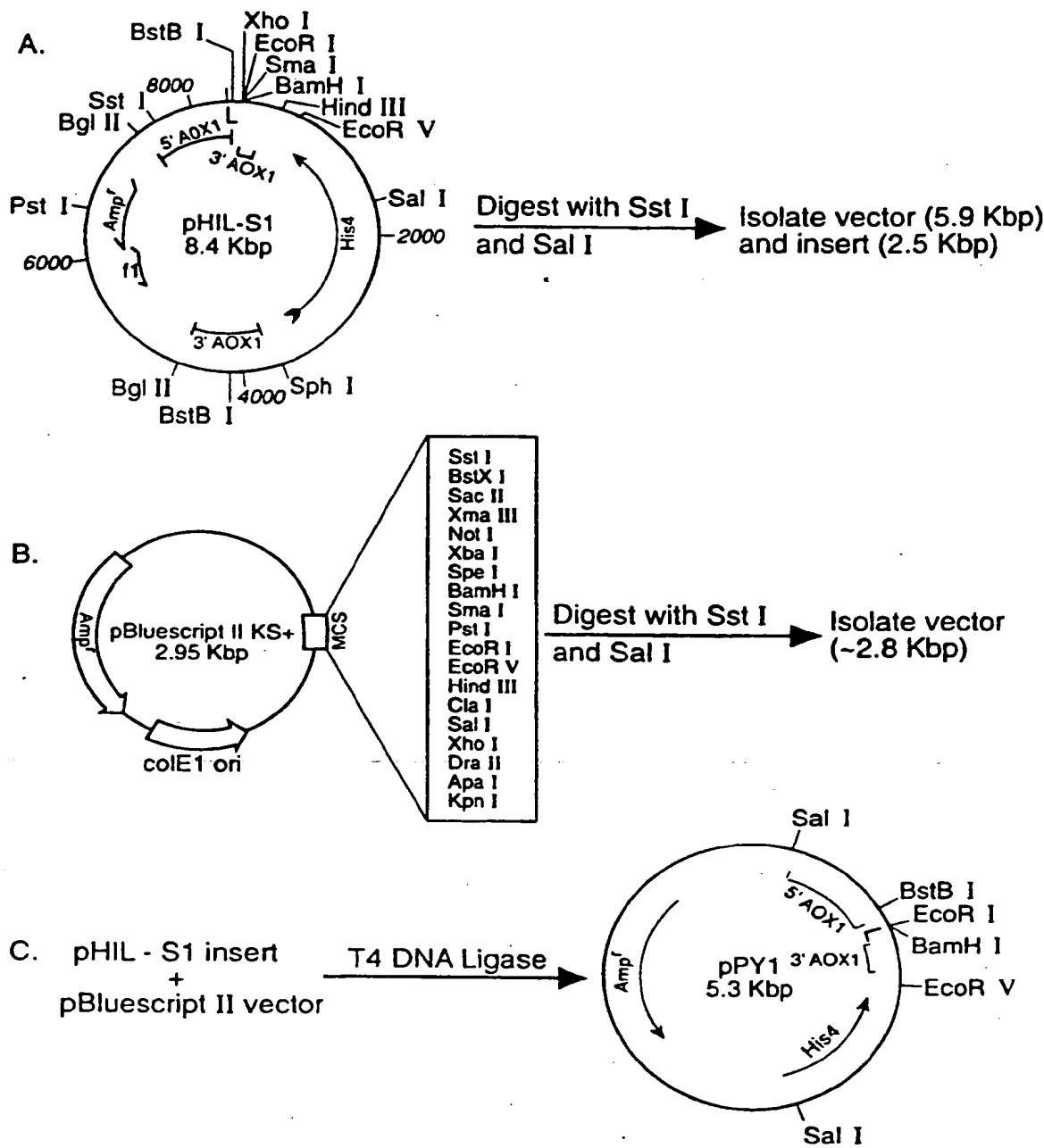
D.

pPY1 —— **Digest with EcoR V** / **Ligate with annealed linker** ——>
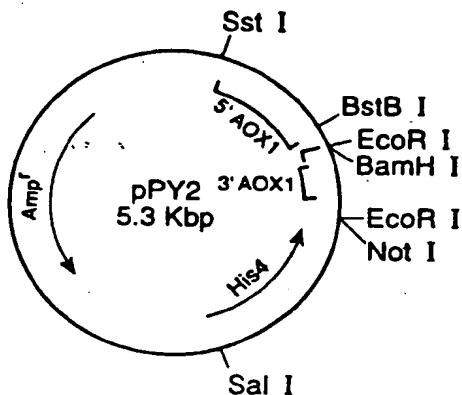
E.

pPY2 —— **Digest with BstB I + BamH I** / **Ligate with CC49 scFv** / **PCR products** ——>
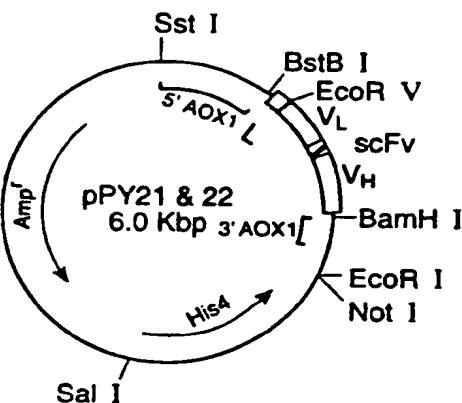
F.

pPY21& / pPY22 —— **Digest with Sst I + Sal I** / **Ligate with pHIL - S1** / **vector fragment from A** ——>
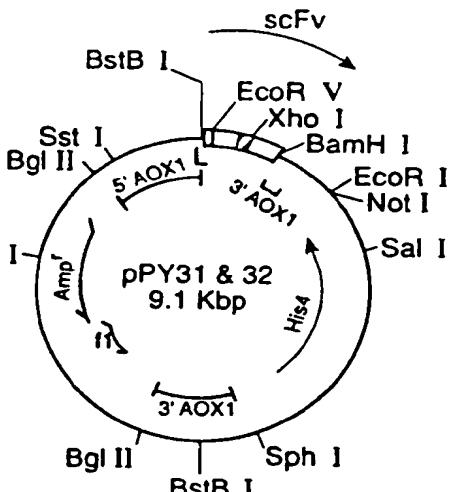
FIGURE 15

Continued

FIGURE 16

DNA AND AMINO ACID SEQUENCE OF CC49 SCFV IN pPY21 & pPY22


```
                              AOX1P
                    TTTAACGACAACTTGAGAAGATC                    BstB I
5'-AOX1---------------------------------- (AAAAAACAAC) TAATTATTC        9


     -22
     Met Lys Tyr Met Leu Leu Gln Ala Phe Leu Phe Leu Leu Ala
GAA ACG ATG AAA TAC ATG CTT TTG CAA GCT TTC CCT TTC CTT TTG GCT        57


                    EcoR V           +1
Gly Phe Ala Ala Lys Ile Ser Ala Asp Ile Val Met Ser Gln Ser Pro
GGT TTT GCA GCT AAG ATA TCT GCT GAC ATT GTG ATG TCA CAG TCT CCA       105


     10
Ser Ser Leu Pro Val Ser Val Gly Glu Lys Val Thr Leu Ser Cys Lys
TCC TCC CTA CCT GTG TCA GTT GGC GAG AAG GTT ACT TTG AGC TGC AAG       153


                         30                            40
Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala
TCC AGT CAG AGC CTT TTA TAT AGT GGT AAT CAA AAG AAC TAC TTG GCC       201


          TAGVLFR2
TGG TAC CAG CAG AAA CCA GG              50
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG       249


                60                            70
Ala Ser Ala Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly
GCA TCC GCT AGG GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA       297


                     80
Ser Gly Thr Asp Phe Thr Leu Ser Ile Ser Ser Val Lys Thr Glu Asp
TCT GGG ACA GAT TTC ACT CTC TCC ATC AGC AGT GTG AAG ACT GAA GAC       345


                         TAGVLCDR3
                          A       T
     90             GT CAG CAG TAT TAT AGC TAT CC
Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
CTG GCA GTT TAT TAC TGT CAG CAG TAT TAT AGC TAT CCC CTC ACG TTC       393


               110        Hind III                   120
Gly Ala Gly Thr Lys Leu Val Leu Lys Leu Ser Ala Asp Asp Ala Lys
GGT GCT GGG ACC AAG CTG GTG CTG AAG CTT AGT GCG GAC GAT GCG AAA       441


                         130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG       489


     Xbo I    140                            150
Asp Leu Glu Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
GAC CTC GAG GTT CAG TTG CAG CAG TCT GAC GCT GAG TTG CTG AAA CCT       537
```


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FIGURE 16 Continued

```
                                    160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr        585
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT

    170                                          180            A
Asp His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu        633
GAC CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA

        TAGVHCDR2    190                                 200
TGG ATT GGA TAT TTT TCT CC
Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu       681
TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG

                                    210
Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr       729
AGG TTC AAG GGG AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT

            220                                  230
Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr       777
GCC TAC GTG CAG CTC AAC AGC CTG ACA TCC GAG GAT TCT GCA GTG TAT

                                    240
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser       825
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA

        250                                         260
A.  Val Thr Val Ser Pro Glu Asp Pro Glu Asp Tyr Asp *** ***          867
    GTC ACC GTC TCC CCT GAA GAC CCT GAA GAC TAT GAC TAA TAG

        250
B.  Val Thr Val Ser Tyr *** ***                                       846
    GTC ACC GTC TCC TAC TAA TAG

BamH I
GGATCCTTAG---------------------------------------AOX1 TERMINATOR-3' 877/856
```

RELATIVE ACTIVITY OF CC49 SCFV SPECIES
PICHIA PASTORIS CULTURE SUPERNATANTS

FIGURE 17

CLONES 1-36: pPY31
CLONES 37-71: pPY32
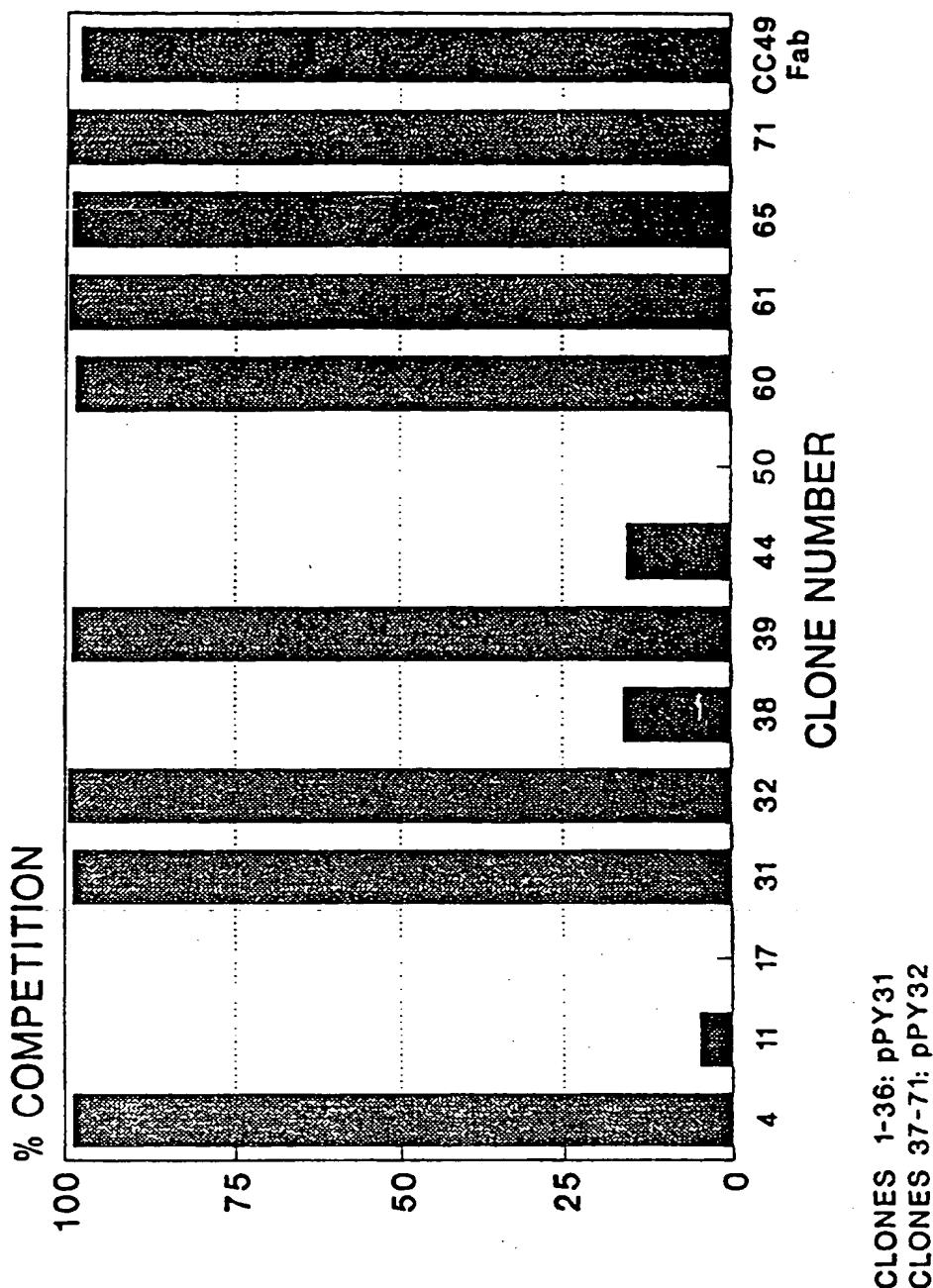
GEL FILTRATION & SDS - PAGE ANALYSIS OF *PICHIA* CC49 scFv
pI = 5.2 SPECIES
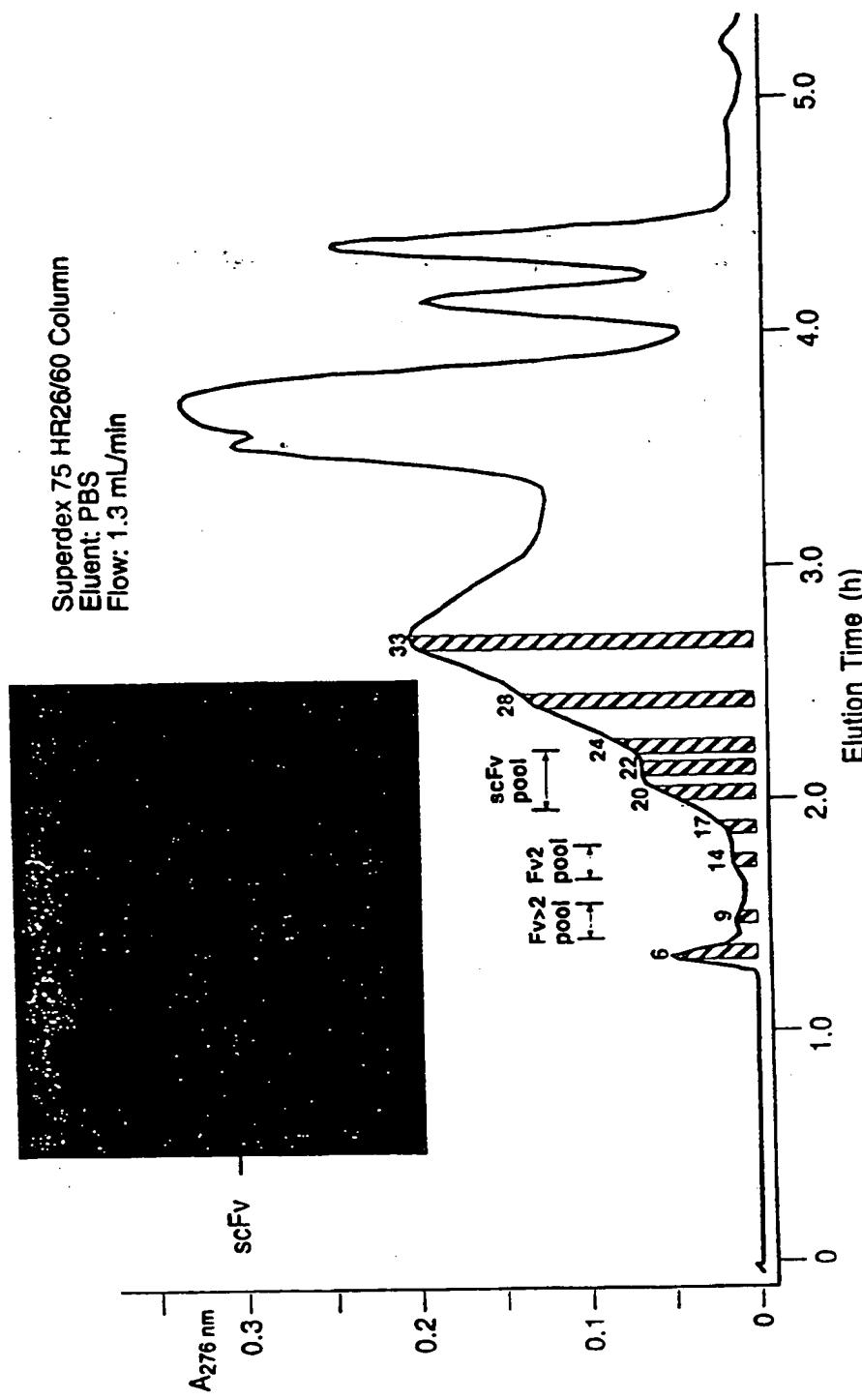
FIGURE 18

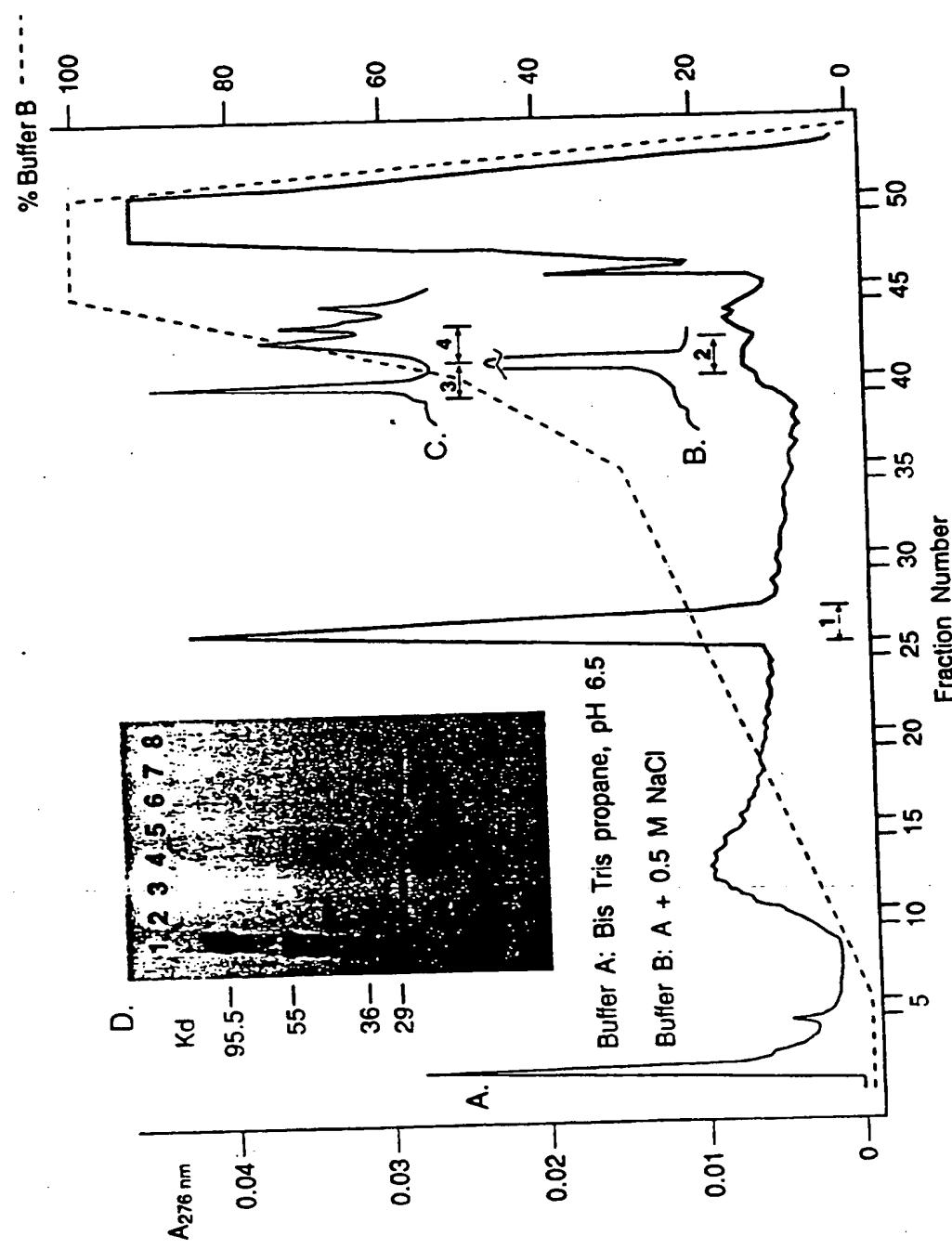ANION EXCHANGE CHROMATOGRAPHY & SDS-PAGE OF
PICHIA CC49 scFv SPECIES

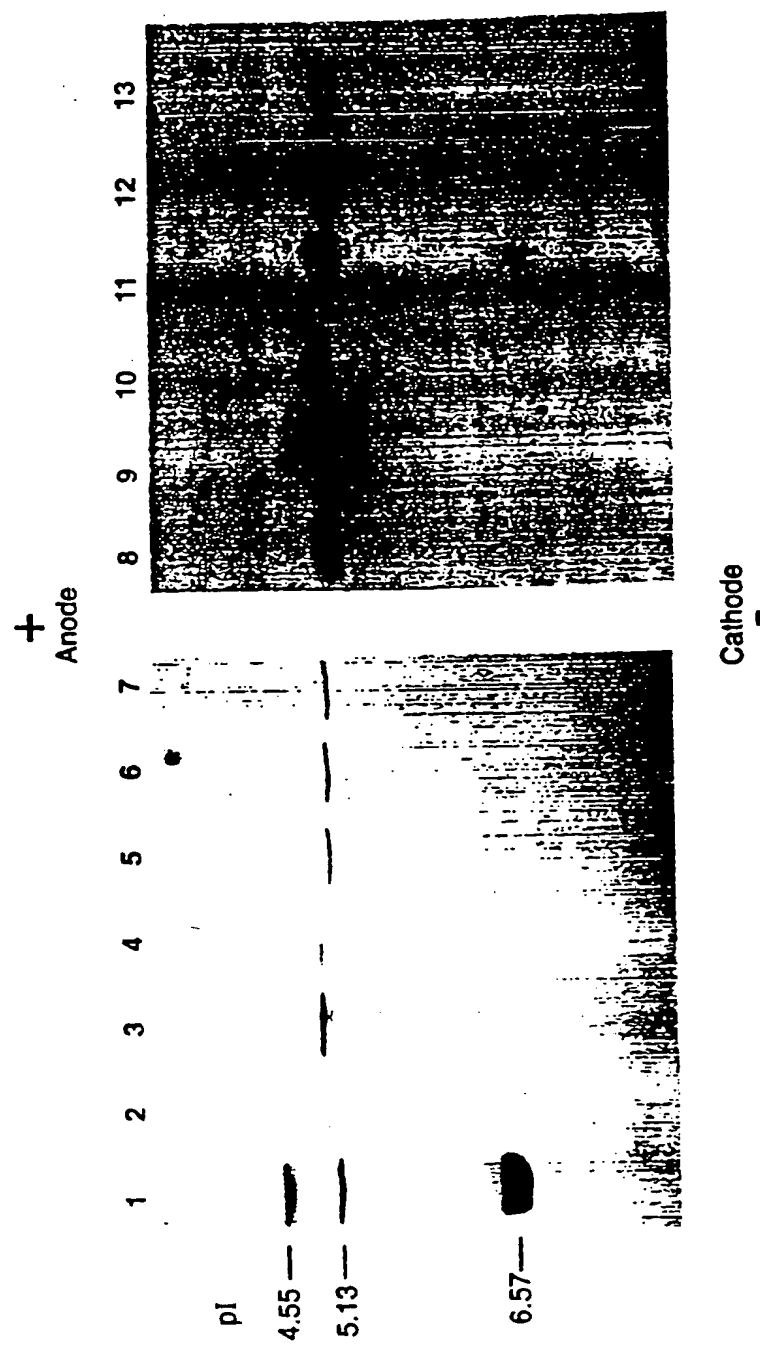
FIGURE 19

IEF & WESTERN OF *PICHIA* CC49 SCFV SPECIES

FIGURE 20
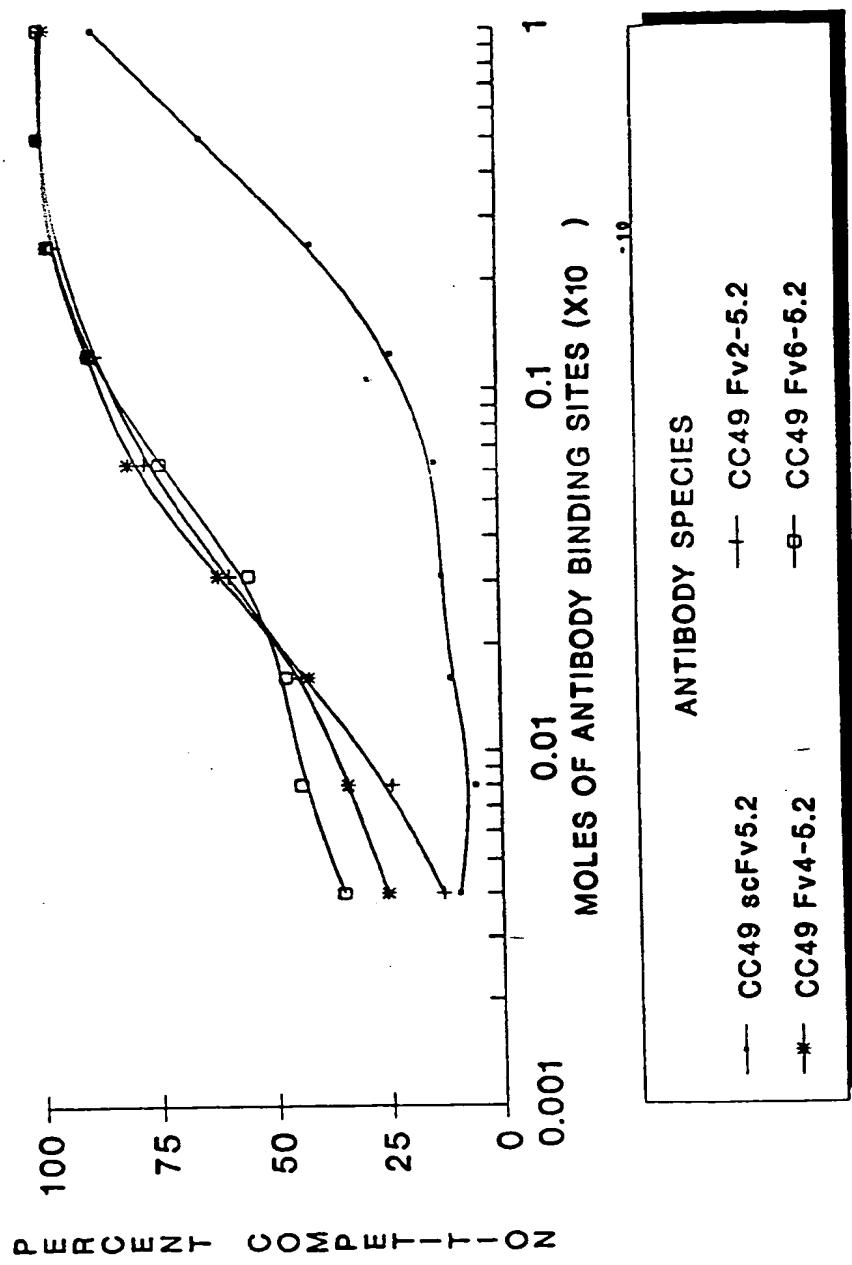
SCFV & MULTIMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG

FIGURE 21

SCFV & MULTIMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG
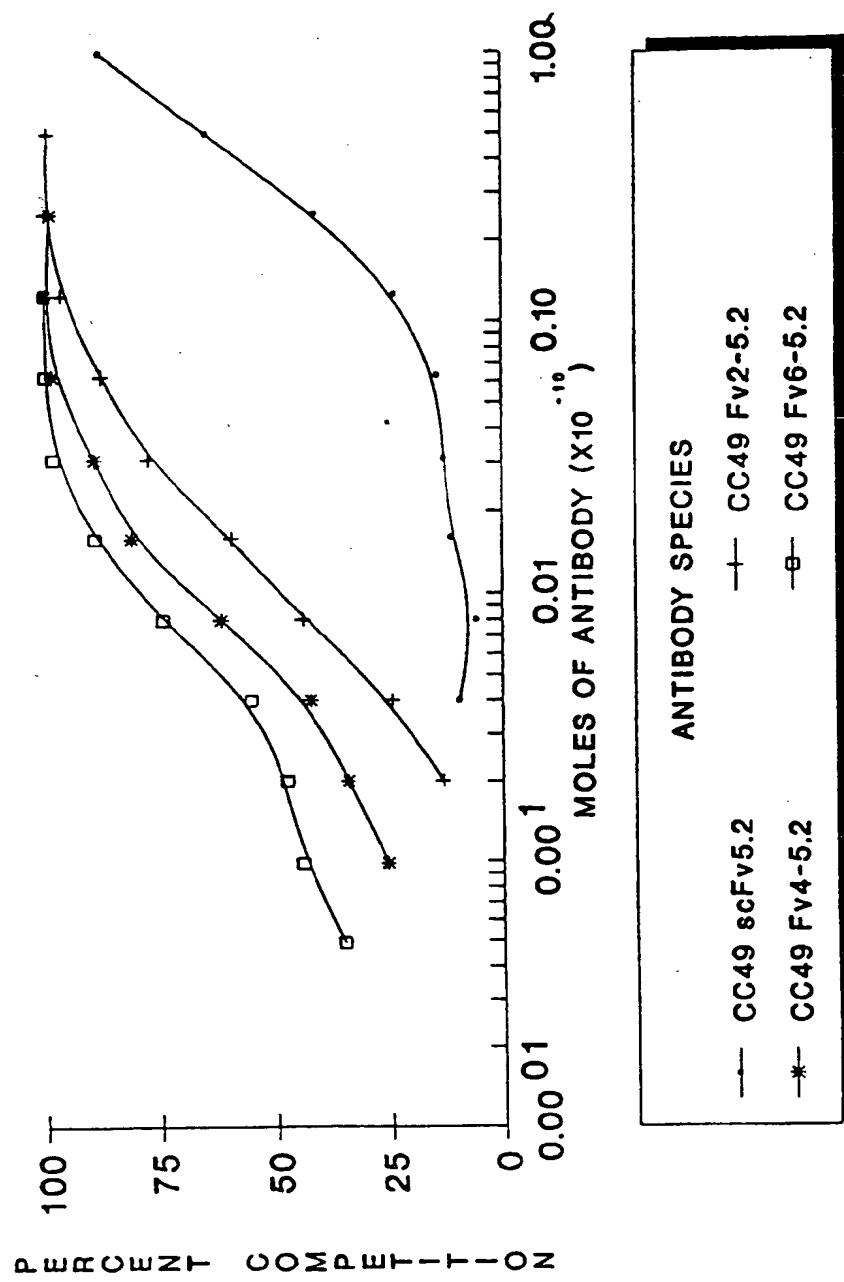
FIGURE 21A

CONSTRUCTION OF pSC49FLAG
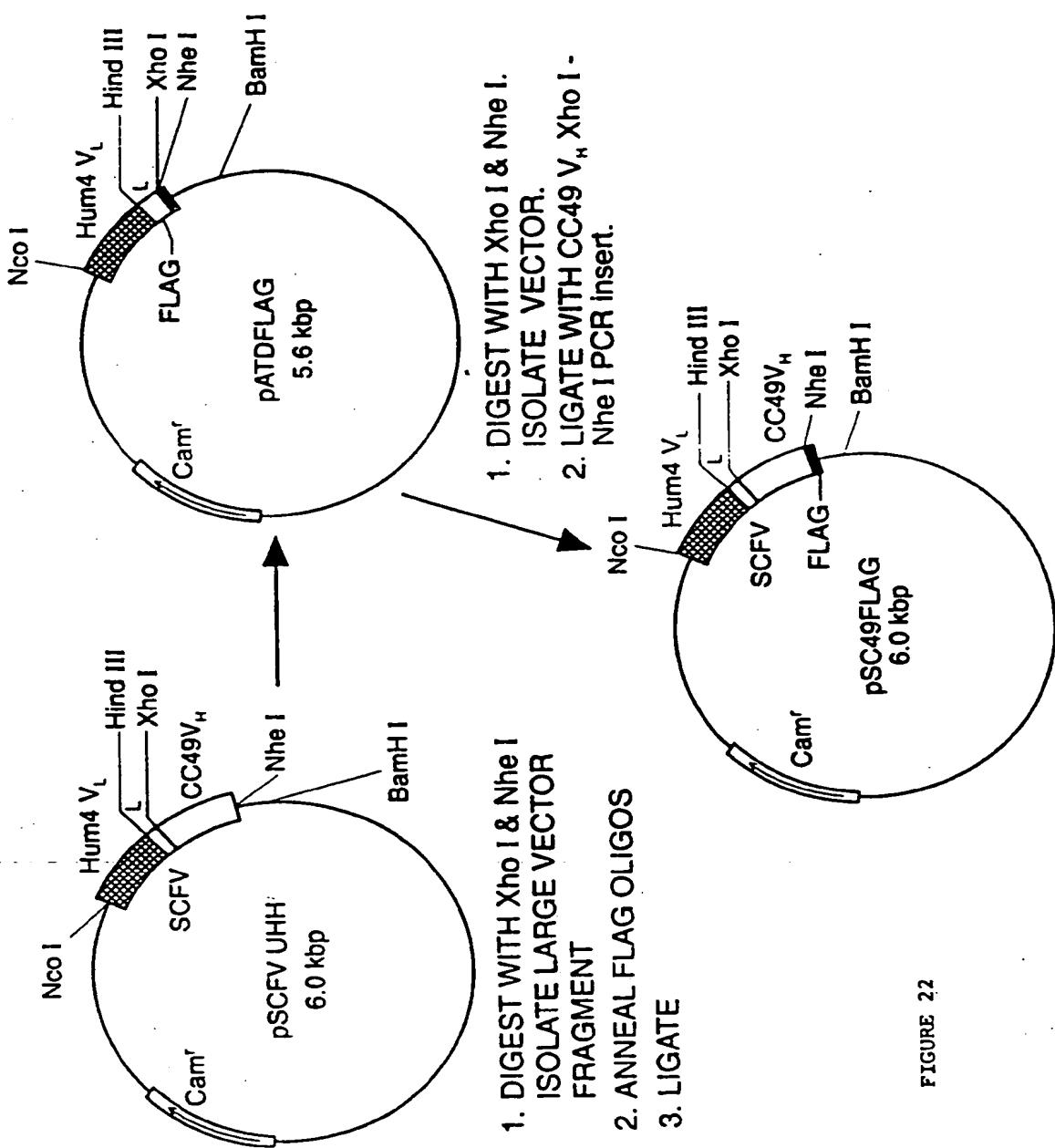
FIGURE 22

FIGURE 23

DNA AND AMINO ACID SEQUENCE IN pATDFLAG

```
                                Cla I    EcoR I
5'-C TCA TGT TTG ACA GCT TAT CAT CGA TGA ATT CCA TCA CTT CCC TCC        46

   GTT CAT TTG TCC CCG GTG GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA       94

   ACG GTT GCA TTT AAA TCT TAC ATA TAT AAT ACT TTC AAA GAC TAC ATT       142

   TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT ACG TAC CAA TTA       190

   TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA AGA       238

   GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG       286

      -22
      Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
   ATT TTG ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA       334

               Nco I        +1
   Leu Ala Ala Gln Pro Ala Met Ala Asp Ile Val Met Thr Gln Ser Pro
   CTC GCT GCC CAA CCA GCC ATG GCC GAC ATC GTG ATG ACC CAG TCT CCA       382

      10                                    20
   Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Lys
   GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC TGC AAG       430

                  30                                        40
   Ser Ser Gln Ser Val Leu Tyr Ser Ser Asn Asn Lys Asn Tyr Leu Ala
   TCC AGC CAG AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT       478

                              50
   Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp
   TGG TAC CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG       526

            60                                        70
   Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
   GCA TCT ACC CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG       574

                              80
   Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp
   TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT       622

            90                                100
   Ala Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
   GCG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC ACT TTC       670

                  110             Hind III              120
   Gly Gly Gly Thr Lys Val Val Ile Lys Leu Ser Ala Asp Asp Ala Lys
   GGC GGA GGG ACC AAG GTG GTG ATC AAG CTT AGT GCG GAC GAT GCG AAA       718
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FIGURE 23 Continued

```
                                    130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG      766


    Xho I              Nhe I
Asp Leu Glu            Ala Ser Asp Tyr Lys Asp Asp Asp Asp Lys ***
GAC CTC GAG ACAATGTCGCT AGC GAC TAC AAG GAC GAT GAT GAC AAA TAA      816


AAA CCT AGC GAT GAA TCC GTC AAA ACA TCA TCT TAC ATA AAG TCA CTT      864
            PENPTSEQ: ← G TTT TGT AGT AGA ATG TAT TTC


GGT GAT CAA GCT CAT ATC ATT GTC CGG CAA TGG TGT GGG CTT TTT TTG      912


TTT TCT ATC TTT AAA GAT CAT GTG AAG AAA AAC GGG AAA ATC GGT CTG      960


CGG GAA AGG ACC GGG TTT TTG TCG AAA TCA TAG GCG AAT GGG TTG GAT     1008


            BamH I
TGT GAC AAA ATT CGG ATC C-3'                                        1027
```

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FIGURE 24

DNA AND AMINO ACID SEQUENCE OF pSC49FLAG

```
                        Cla I    EcoR I
5'-C TCA TGT TTG ACA GCT TAT CAT CGA TGA ATT CCA TCA CTT CCC TCC      46

GTT CAT TTG TCC CCG GTG GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA       94

ACG GTT GCA TTT AAA TCT TAC ATA TAT AAT ACT TTC AAA GAC TAC ATT      142

TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT ACG TAC CAA TTA      190

                    PENPR1: AAC ACT GTA GGG ATA GTG GAA →
TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA AGA      238

GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG      286
                  PENPR2: ← TAT AAG TTT GCC TCC CTC TG


        -22
        Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
ATT TTG ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA      334

            Nco I        +1
Leu Ala Ala Gln Pro Ala Met Ala Asp Ile Val Met Thr Gln Ser Pro
CTC GCT GCC CAA CCA GCC ATG GCC GAC ATC GTG ATG ACC CAG TCT CCA      382

    10
Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Lys
GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC TGC AAG      430

                                                              40
Pro Ser Gln Ser Val Leu Tyr Ser Ser Asn Asn Lys Asn Tyr Leu Ala
CCC AGC CAG AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT      478

TAGVLFR2:
TGG TAC CAG CAG AAA CCA GG →        50
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp
TGG TAC CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG      526

            60                                      70
Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GCA TCT ACC CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG      574

                        80
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT      622

    90                                      100
Ala Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
GCG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC ACT TTC      670

                    110        Hind III              120
Gly Gly Gly Thr Lys Val Val Ile Lys Leu Ser Ala Asp Asp Ala Lys
GGC GGA GGG ACC AAG GTG GTG ATC AAG CTT AGT GCG GAC GAT GCG AAA      718
```

FIGURE 24 Continued

```
                UHVHSEQ:
        GAT GCT GCG AAG AAG GAT GAC G → 130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG      766

    Xho I    140
Asp Leu Glu Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
                                                    150
GAC CTC GAG GTT CAG TTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT      814

                        160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT      862

    170                                    180    TAGVHCDR2: A
Asp His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu
GAC CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA      910

TGG ATT GGA TAT TTT TCT CC →                        200
Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu
TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG      958

                        210
Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr
AGG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT      1006

    220                                    230
Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT      1054

                TAGVHFR4: AC TGG GGT CAA GGA ACC TCA
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA      1102

G → 250                Nhe I                260
Val Thr Val Ser Ser Ala Ser Asp Tyr Lys Asp Asp Asp Asp Lys ***
GTC ACC GTC TCC TCA GCT AGC GAC TAC AAG GAC GAT GAT GAC AAA TAA      1150

AAA CCT AGC GAT GAA TCC GTC AAA ACA TCA TCT TAC ATA AAG TCA CTT      1198

GGT GAT CAA GCT CAT ATC ATT GTC CGG CAA TGG TGT GGG CTT TTT TTG      1246

TTT TCT ATC TTT AAA GAT CAT GTG AAG AAA AAC GGG AAA ATC GGT CTG      1294

CGG GAA AGG ACC GGG TTT TTG TCG AAA TCA TAG GCG AAT GGG TTG GAT      1342

        BamH I
TGT GAC AAA ATT CGG ATC C-3'                                        1361
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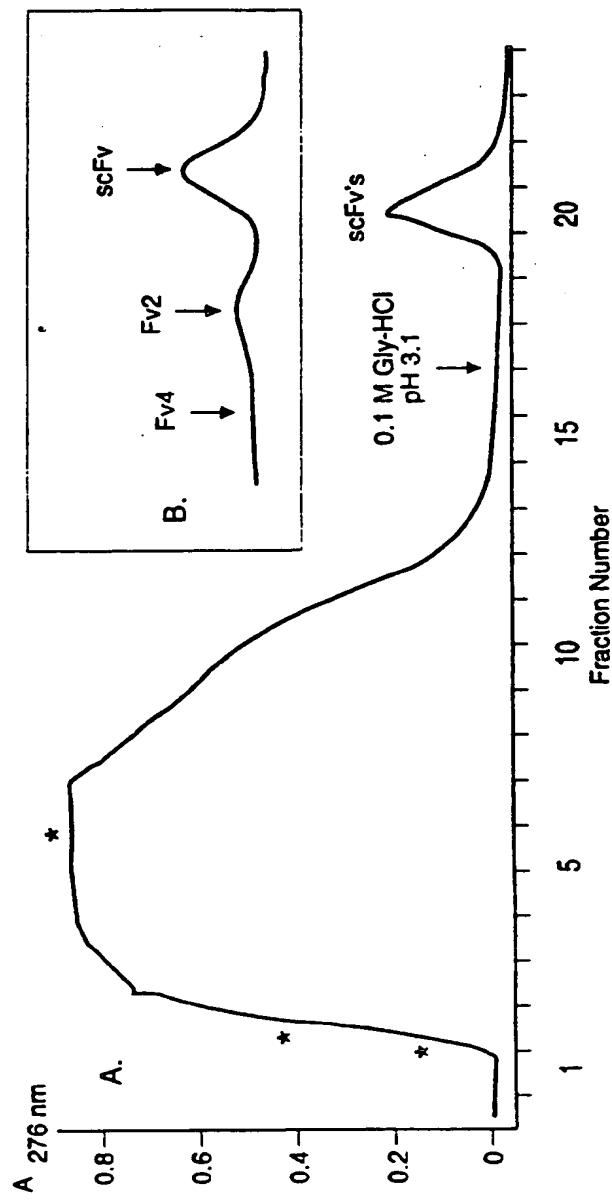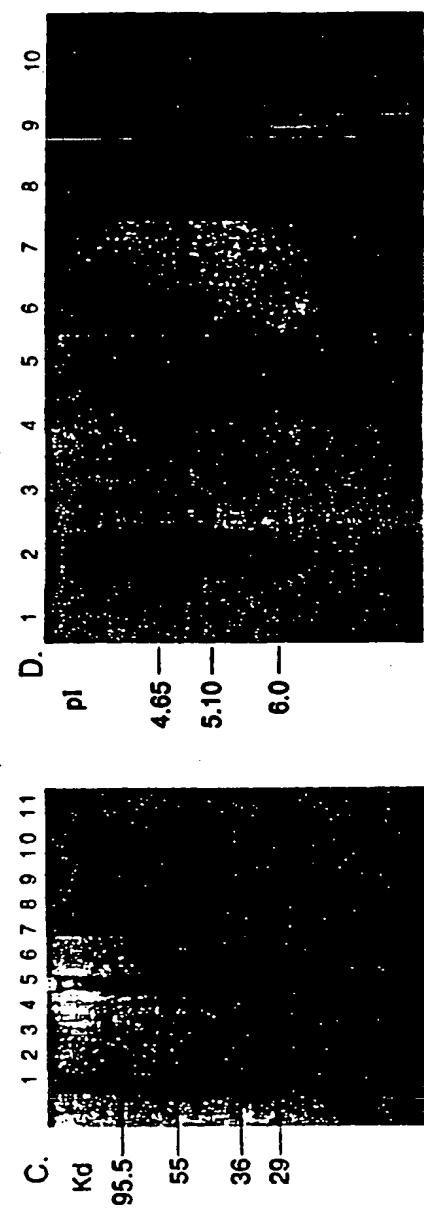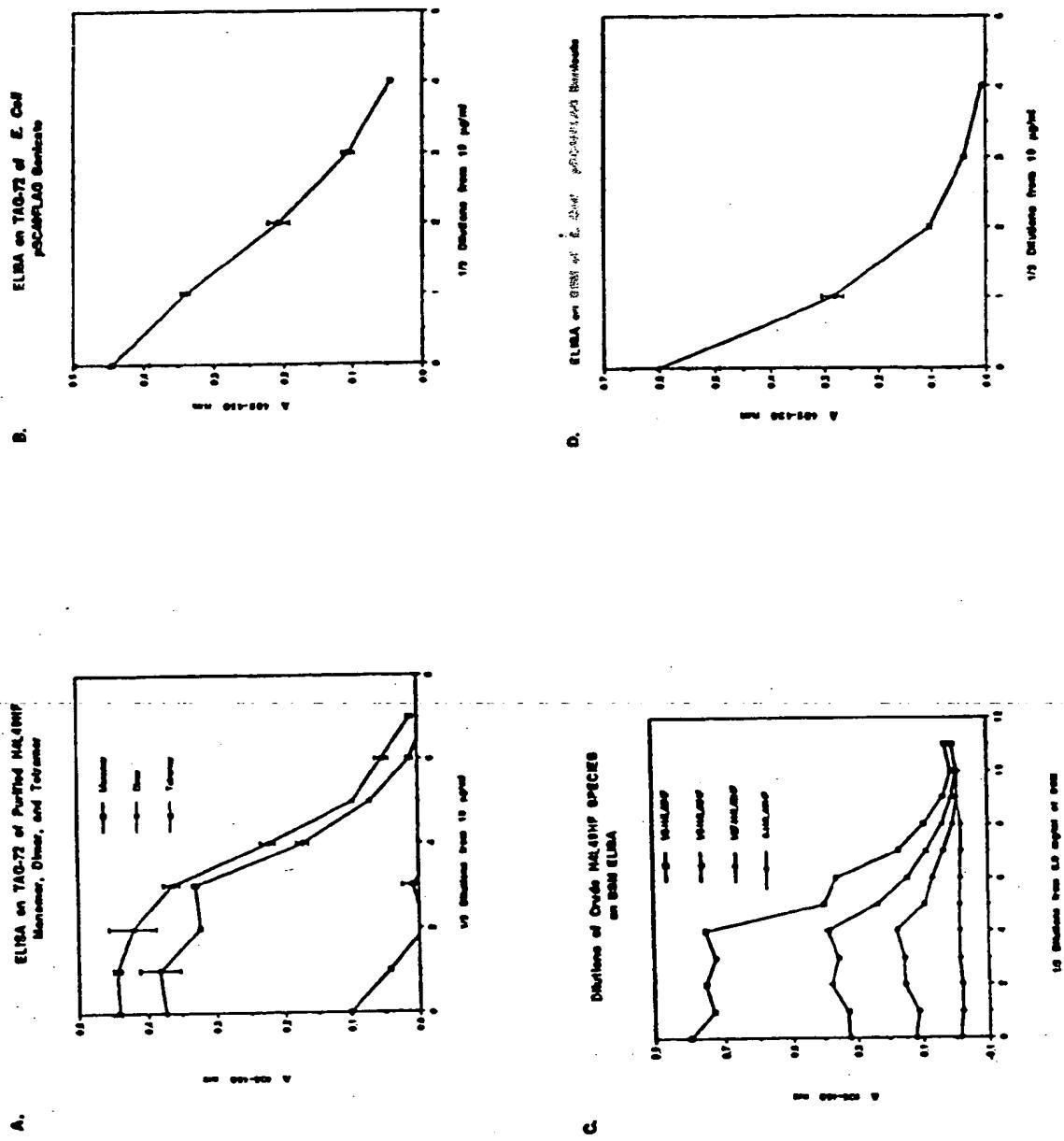PURIFICATION AND CHARACTERIZATION OF H4L49HF scFv's

FIGURE 25

FIGURE 26

SCFV & MULTIMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG

ANTIBODY SPECIES

—— H4L49HF scFv   —+— H4L49HF Fv2   —*— H4L49HF Fv4

FIGURE 27

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SCFV & MULTIMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG

FIGURE 28

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PCT

(51) Internati nal Patent Classification 5 :		(11) International Publication Number:	**WO 94/04691**
C12N 15/62, 15/13, C07K 15/00 A61K 39/395, 47/48, 49/02 G01N 33/577, 33/53	A3	(43) International Publication Date:	3 March 1994 (03.03.94)

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(30) Priority data:
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(71) Applicant: THE DOW CHEMICAL COMPANY [US/US]; 2030 Dow Center, Abbott Road, Midland, MI 48640 (US).

(72) Inventors: MEZES, Peter, S. ; 5101 Oakridge Drive, Midland, MI 48640 (US). RICHARD, Ruth, A. ; 5111 Plainfield Street, Midland, MI 48640 (US). AFFHOLTER, Joseph, A. ; 823 E. Sanford Road, Midland, MI 48640 (US). KOTITE, Nicolas, J. ; 5704 Woodbridge Lane, Midland, MI 48640 (US).

(74) Agent: BROOKENS, Ronald, G.; The Dow Chemical Company, Patent Department, P.O. Box 1967, Midland, MI 48641-1967 (US).

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Published
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(88) Date cf publication of the international search report:
31 March 1994 (31.03.94)

(54) Title: DIMER AND MULTIMER FORMS OF SINGLE CHAIN POLYPEPTIDES

CONSTRUCTION OF *E.COLI* SCFV EXPRESSION PLASMIDS CONTAINING HUM V_LIV

(57) Abstract

The present invention discloses novel proteins which are dimers and multimers of single chain antibodies. The single chain antibodies contain polypeptides of two domains derived from the variable domains of antibodies which are joined by a peptide linker. The dimers and multimers are formed by non-covalent linking of the single chain polypeptides. Uses include all those appropriate for monclonal and polyclonal antibodies and fragments thereof, including use as a bispecific antigen-binding molecule.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5   C12N15/62   C12N15/13   C07K15/00   A61K39/395   A61K47/48
        A61K49/02   G01N33/577   G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5   C12N   C07K   A61K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 19739 (CELLTECH LIMITED) 26 December 1991 see claims ---	1-14,17
A	US,A,4 946 778 (LADNER ET AL.) 7 August 1990 see claims ---	1-17
P,X	WO,A,93 11161 (ENZON, INC.) 10 June 1993 see claims ---	1-14,17
P,X	WO,A,93 15210 (MERCK PATENT GMBH) 5 August 1993 see claims see page 13, line 17 - page 16, line 22 ---	1-4,13, 14

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 January 1994	1 1 -02- 1994

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer NOOIJ, F

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Inter **mal** Application No

PCT/US 93/07918

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP,A,O 506 124 (TANOX BIOSYSTEMS, INC.) 30 September 1992 see page 6, column 9, line 4 - line 52 see claims ---	1-14,17
P,X	WO,A,93 12231 (DOW CHEMICAL (AUSTRALIA) LIMITED) 24 June 1993 see claims -----	1-17

4

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INTERNATIONAL SEARCH REPORT

.nformation on patent family members

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EP-A-0506124	30-09-92	AU-B-	640863	02-09-93
		AU-A-	1299292	15-10-92
		JP-A-	5117164	14-05-93
WO-A-9312231	24-06-93	AU-B-	9058291	19-07-93